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13. ABSTRACT ***(Maximum 200 Words)***
Our progress on prostate cancer (PCa) to date has centered on the application of spectral karyotyping (SKY) and comparative genomic hybridization (CGH) to study consistent chromosomal changes. This work has resulted in three manuscripts (Appendices 1-3). We are now focusing on different cellular mechanisms that could lead to chromosomal aberration and consequent alterations of gene expression. To study early changes in the tumorigenic process, we have analyzed high-grade prostrate intraepithelial neoplasia (HPIN) using interphase flrorescence in situ hybridization (FISH). Our results suggest that the overall incidence of numerical abnormalities of chromosomes 7, 8 and 10 are more common in HPIN from patients who later showed carcinoma in a subsequent follow-up biopsy. Our recent results suggest that chromosomal instability (CIN) is a feature of Pca and that HPIN foci that progress to and/or are situated adjacent to carcinoma foci are more likely to express CIN than benign HPIN. To further study such early changes using molecular approaches we have developed degenerate oligonucleotide primed DOP-PCR methodologies to allow us to apply CGH methods using DNA microdissected from small foci of tumor or from PIN lesions at the time cancer arises in PCa patients. In parallel we are now studying alterations to gene expression and gene copy number in PCa using the most up-to-date CGH microarray methods.

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Jeremy A. Squire, Ph.D.

PI - Signature Date

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INTRODUCTION

Prostate cancer (PCa) has become the most commonly diagnosed cancer in men in North America with a mortality rate second only to lung cancer. As humans develop a longer life expectancy, the negative impact of PCa will rise dramatically. For example, at autopsy, a very large fraction (up to 30%) of elderly men in some cultures have evidence of PCa although there had been no clinical symptoms during life. With a longer life expectancy an increasing number of men will develop PCa and at present, the treatment of this disease is particularly problematic. Despite its prevalence, our understanding of the cellular and genetic basis for prostate tumorigenesis and metastasis remains limited. PCa appears to be distributed across a very broad spectrum of aggressiveness, and there are no reliable predictors of tumor behavior. A critical issue in the management of PCa has been the lack of DNA-based markers for early detection and subsequent cure. Since PCa is curable when it is organ-confined, but is not easily curable when it has spread beyond the prostate, prognostic indicators of aggressive disease are essential for increased survival. Acquisition of genetic mutations of oncogenes and tumor suppressor genes underlie most cancers, and the challenge for cancer researchers is to isolate these causative genes and to determine their role in the malignant process. There are now many examples of human cancers where a molecular genetic approach has yielded valuable information that helps with the management of cancer patients. Chromosomal analysis of tumors has proven to be the best place to commence a strategy of identification of genes associated with specific tumors. In general, classical cytogenetic techniques have not been effective in detecting chromosomal aberrations in PCa due to the intratumor heterogeneity, and poor cell viability and chromosome quality. This report will address our progress in the context of the 'Statement of work' and the 'Future work in the second phase of the proposal' sections of our original grant application. Our working hypothesis is that PCa-specific chromosomal changes will lead to loss of function of tumor suppressor gene(s) and/or activation of oncogenes and that clues concerning the location of such changes will be detected by a multipronged strategy using the most up to date molecular cytogenetic screening methods. Our experiments have been focusing on the critical change(s) present in early human PCa and ones that are associated with poor outcome.

PROGRESS TO DATE

Aim 1."Evidence for specific chromosome translocation(s) /rearrangement(s) in early PCa?"

1. Spectral Karyotyping (SKY) of PCa cell lines

We are the first group in North America to apply SKY methodologies to PCa (Appendices 1 and 2). In Appendix 1 our SKY analysis of PCa cell lines identified a large number of structural aberrations suggesting there was an underlying chromosomal instability and subsequent accumulation of cytogenetic alterations that confer a selective growth advantage. The high involvement of centromeric rearrangements in these lines indicates a potential role for mitotic irregularities associated with the centromere in PCa tumorigenesis. These observations were in agreement with the multistep model of accumulated hits in PCa tumorigenesis and suggested an increasing importance in understanding the role of the centromere in PCa tumorigenesis

In Appendix 2, we collaborated with Dr. J. Macoska's laboratory to study short-term PCa cultures her laboratory has developed. We utilized a combination of conventional and SKY techniques and allelotype analysis to assess numerical and structural chromosomal alterations in two normal- and three malignant-derived prostate epithelial cell lines immortalized with the E6 and E7 transforming genes of human papilloma virus (HPV) 16 or the Large T antigen gene of simian virus 40 (SV40). These studies revealed trisomy for chromosome 20 and rearrangements involving chromosomes 3, 8, 10, 18, 19, 20 or 21. In addition, the four HPV-immortalized cell lines exhibited extensive duplications or translocations involving the 11q13q22,q23 chromosomal region. Interestingly, allelotyping data disclosed loss of 8p sequences in two of the five cell lines, and the spectral karyotyping data revealed that the loss of 8p sequences in these tumor-derived cell lines was directly due to i(8q) chromosome formation and/or other structural alterations of chromosome 8. The allelotyping (performed by Dr. Macoska's laboratory) showed that molecular changes not apparent by cytogenetic methods were present in these PCa cell lines. This study provided intriguing evidence that 8p loss in human prostate tumors could, in some cases, result from complex structural rearrangements involving chromosome 8.

Furthermore, this data provided the first direct evidence that such complex structural rearrangements sometimes includes i(8q) chromosome formation. To determine whether similar cytogenetic aberrations were present in patient tumors we have evaluated tumor tissue derived from 15 surgical resections using different modifications of conventional cytogenetic methods for solid tumors. Firstly, we varied the constituents of the culture media using suggestions from Dr. D. Peehl (1) and investigators working in the laboratory of Dr. S. Heim (2). Secondly, we have used an irradiated murine feeder cell line S17 as a source of paracrine cytokines to stimulate epithelial cell growth (3). While this substrate appeared to produce improved growth it was technically difficult to process additional quiescent cells. We are presently using the following media with good success:

F12K media supplemented with:
15% FCS
3 mg/ml L-glutamine
100 U/ml penicillin
100 µg/ml streptomycin
10 ng/ml epidermal growth factor
10^{-6} M hydrocortisone
20 ng/ml dihydrotestosterone
1 µg/ml sodium selenite

Fixed cells are being analyzed for the presence of structural chromosome aberrations. In keeping with the findings of other (2) a low percentage of cells have cytogenetic aberrations. In appendix 4 current findings with patient tumors are presented. In parallel DNA from the same tumors have been analyzed by CGH and interphase cytogenetic methods (see Section 2.a and Appendix 3). In summary, we have fully characterized the chromosomal constitution of 9 PCa cell lines to date using SKY methods and our studies draw attention to structural and numerical alterations of chromosome 8 and suggest that high resolution CGH (see Section 3) will be an appropriate approach for detailed mapping and positional cloning in these chromosomal regions.

Aim 2. "Evidence for consistent predictive chromosome instability (numerical and structural gains and/or losses) in pre-neoplastic and/or early preinvasive carcinoma"

2.(a) CGH Analysis of Patient samples

In Appendix 3 we have applied CGH and interphase FISH to fourteen early stage PCa specimens in order to: (1) evaluate the utility of CGH for examining bulk-extracted genomic DNA from early stage PCa specimens; (2) identify all regions of chromosomal gain and loss present in each patient sample; (3) determine whether there are any consistent genomic dosage changes, common to the patient cohort; and (4) verify any aberrations found by CGH using interphase FISH. CGH and interphase FISH methodologies were used that are well established in this laboratory (4, 5, 6, 7).

Our analysis demonstrated that copy number changes as detected by CGH were not a feature of the majority of the tumors studied, with only 14% of tumors having alterations. However, our application of interphase FISH that permitted an assessment of genotypic heterogeneity on a cell-by-cell basis revealed heterogeneity at the CEP8/*MYCC* loci in a larger subset of the study group. Our conclusions from this study are: (1) that there is a lower than expected frequency of genomic copy number alterations in early PCa; (2) that genotypic heterogeneity potentially due to a chromosomal instability (CIN) phenotype may be a feature of the early phases of PCa tumorigenesis; (3) that alterations to the copy number of chromosome 8 are the most frequent changes in early PCa; and (4) that normal stromal cell contamination could be reducing the sensitivity of the CGH method.

While several CGH studies using primarily pT3 tumors have previously shown that CGH copy number changes are relatively frequent in PCa (8, 9, 10, 11, 12) (25% tumors), our analysis found a lower overall frequency (14%). Our study group comprised of only low stage (pT1-T2) tumors suggesting that a different experimental approach was required to study early chromosomal changes in PCa. One explanation is that the early alterations to the genome or PCa tumors are submicroscopic and do not span sufficient regions to be detected at the level of resolution of traditional CGH. To address this possibility we are applying CGH microarray methods (see Section 3) using probes derived from the short arm of chromosome 8. However

a more immediate concern is that it is possible that the heterogeneity in the genome of the microfoci of tumors and/or the genome of the stromal tissues significantly "dilutes" any specific alterations to undetectable levels. To overcome this potential problem we have established degenerate oligonucleotide primed (DOP)-PCR for the amplification of the whole genome from as little as 30 pg of DNA (Appendix 6). In addition, we have developed a laser capture microdissection (LCM) technique to isolate a homogenous population of epithelial cells from an H&E-stained paraffin section of PCa tumor (Appendix 6). We are presently extracting DNA from such preparations and will generate more homogeneous probes by DOP-PCR for CGH analysis of microfoci of tumors dissected from pT1-T2 tumors. We will apply LCM/DOP-PCR to re-examine archived paraffin embedded material from patient samples analyzed by bulk extraction methods (Appendix 3). Furthermore, if successful, LCM and DOP-PCR methods will be applied to compare several foci within a same tumor to address the issue of heterogeneity in greater detail.

To study the CIN phenotype in greater depth and to understand how this type of aberration may be a useful predictor of poor outcome in PCa, we have studied the three cell lines utilized in Appendix 1 to quantify the number of centromeres as an index of aneusomy. Our preliminary results indicate that there is a low level of CIN in the three cell lines (manuscript in preparation). We plan to extend this study to include patient samples.

2.(b) Identification of numerical chromosomal changes in high-grade prostrate intraepithelial neoplasia (HPIN) as a predictor of carcinoma.

Concurrently with the above CGH studies we have been retrospectively analyzing numerical chromosomal changes by interphase FISH on a cell-by-cell basis on whole mount sections to determine levels of intratumor cytogenetic heterogeneity and to determine whether the assay can be used as an additional predictor of increased risk of carcinoma (Appendix 5). It is well-established that HPIN is the most likely precursor of PCa and its identification in biopsy specimens invariably warrants further searching for invasive carcinoma; however not all cases of HPIN progress to carcinoma. About half of patients with HPIN have carcinoma identified in the second subsequent follow-up biopsies. No available clinical or immunohistochemical or morphological criteria that can be predictive of this progression or association has been documented.

Two groups of patients were used for this retrospective study. The first revealed carcinoma on the follow-up biopsies and the second revealed persistent HPIN and/or other benign lesions. Molecular cytogenetic analysis is being performed to determine whether there is any difference in numerical chromosome copy number in these two groups. To date interphase FISH analysis has been performed on biopsies from 28 patients (12 from the first group and 16 from the second). We have utilized formalin fixed paraffin-embedded tissue sections (5µm). Probes for chromosome 4, 7, 8 and 10 centromeric region have been used as two color FISH experiments and the number of signals have been counted in 100 non-overlapped nuclei. The criteria for chromosomal gain and loss is >8% of cells with >2 signals and >50% of cells with <2 signals respectively. Presently, thirty-three percent of the first group displayed numerical chromosomal aberrations. Only 12.5% of the patients from the second group had chromosomal anomalies. All chromosomal changes were detected in a form of gain and no chromosomal losses have been identified. Overall, the most common anomaly was gain of a chromosome 8, followed by chromosomes 7 and 10. No anomalies have been seen in the adjacent hyperplastic or normal prostate glandular epithelium. Our results so far indicate that although no single numeric chromosomal anomaly could be assigned as a predictor of progression of HPIN to carcinoma, it is evident that the presence of numerical chromosomal abnormalities in chromosomes 7, 8 and 10 are more common in HPIN from patients who showed carcinoma in the subsequent follow-up biopsies. There is no statistically significant difference between the two samples ($p>0.05$) in the overall numeric chromosomal abnormalities for chromosomes 7, 8 and 10. It should be stressed that our sample size is small and that by expanding our study to a larger sample size we will obtain in Phase 2 of the study period should permit us to demonstrate a significant difference. Our results are also important mechanistically since they suggest that chromosomal instability is more common in HPIN foci that progress to and/or are situated adjacent to carcinoma foci.

Aim 3. "Positional mapping to identify putative candidate genes which may be useful as prognostic indicators of early disease."

3. Application of array technologies for fine structure mapping and analysis of gene expression in PCa

In a complementary, but independently funded study, work in our laboratory has recently been examining the differences in gene expression between the tumors derived from the patients who present with disease recurrence and those who remain disease-free following radical prostatectomy. To date, we have identified from screening of the Clontech Atlas Human Cancer array, 4 genes whose over-expression correlates with disease recurrence, including epithelial cell marker protein 1, endonuclease III homologue 1, vascular endothelial growth factor (VEGF) precursor, and IgA1. We have recently adapted this approach to screen by FISH a more comprehensive, broader-spectrum array of 1700 genes and ESTs (Appendix 6), and we are now able to analyze the expanded array panel of 20,000 ESTs. To achieve our goal of positional mapping novel gene(s) associated with early PCa we will use modified microarray methods to perform copy number CGH analysis of ESTs. Since the chromosomal locations of many of the ESTs on this array are already known we will be able to focus on regions of chromosome 8 already implicated as being involved by this study (Appendices 1-3) and others (13). The improved resolution of microarray CGH will allow us to detect small chromosomal deletions that cannot be resolved by CGH analysis of metaphase chromosomes (see Section 2a). In addition, our Institute has arranged to have early access to new sequence data from the Celera Genomics (http://www.celera.com). We will select UniGene EST contig clusters and predicted genes which map to the chromosomal regions 8p12 (D8S505-D8S135), 8p22 (D8S520-D8S552 and MSR-D8S258), and 8p23 (D8S1781-D8S262). For gene prediction and protein analysis we will use standard software (GenScan, SWISS-PROT, GRAIL Genetool, Peptool, etc.) and we will closely work with the Genome Center based at the Hospital for Sick Children (http://www.bioinformatics-canada.org)in this phase of the project.

We will select ESTs at ~50 kb intervals as targets for microarray CGH and will use protocols similar to those used in Appendix 3 and in all previous CGH publications from our laboratory. We will recognize submicroscopic deletions by a signal decrease to ~ 0.5-0.6 by ratio analysis. To confirm such deletions we will use genomic probes that map to the deleted region(s) of chromosome 8 for direct FISH analysis of paraffin sections using methods described in Appendix 5. We anticipate being in a strong position to localize the region(s) and gene(s) involved in the development of PCa

and poor disease outcome as we enter the final phase of our work.

KEY RESEARCH ACCOMPLISHMENTS:

- First SKY paper analyzing PCa (selected as the cover feature for the issue)
- First delineation of chromosome 8 alterations by SKY in PCa cell lines
- Optimization of *in vitro* conditions for short term culture and SKY analysis of patient PCa tissue
- CGH analysis of PCa patient tumors identifies a low frequency of chromosomal copy number aberration in bulk extracted tissue
- Development of laser capture microdissection and DOP-PCR methods for CGH analysis of tumor microfoci.
- Characterization of chromosomal instability (CIN) phenotype in PCa cell lines and patient samples.
- Recognition that CIN is more common in HPIN foci that progress to and /or is situated adjacent to carcinoma foci.
- Development of glass slide-based microarray for analysis of gene copy number and expression by CGH

REPORTABLE OUTCOMES:

- 2 papers in press (Appendices 1 and 2).
- 2 manuscripts in preparation (Appendix 3 and section 2a).
- 9 abstracts.
- Two graduate students are working on this project. Dr. Jaudah Al-Maghrabi is a M.D. Pathologist on a training fellowship from Saudi Arabia. He will complete his M.Sc. based on the data presented in Appendix 5. His fellowship support derives from Saudi Arabia.
- Ben Beheshti recently reclassified to become a predoctoral student. His student stipend is supported by the Paul Starita Fellowship and a University of Toronto Open Scholarship.
- Dr. Paul Park derives part of his stipend from the American Foundation for Urological Diseases. Dr. Park is the first Canadian to receive an AFUD scholarship award for his work on prostate cancer.
- Development of chromosome 8 EST database for gene discovery/ LOH analysis.
- Application to National Cancer Institute of Canada (NCIC) for research grant supporting application of microarray analysis to detect prognostic differential gene expression in PCa.

- Two month academic research visit from Dr. Monica Nunes (University of Sao Paulo) to study HPIN in PCa.

CONCLUSIONS:

Our studies have demonstrated the value of using the most sensitive molecular detection methods to study DNA alterations in early PCa lesions. SKY has shown that long-term cell lines have a much greater complexity of aberration than short-term cultures. In contrast CGH analysis suggests that the karyotype may be relatively simple. By performing a detailed interphase FISH on HPIN we have shown that the earliest probable change in PCa is the onset of a CIN phenotype within HPIN lesions. Phase 2 of our study will allow us to determine whether this finding is statistically significant and go on to develop this assay so that it can be offered as part of the routine assessment of patient samples in the future. In addition we will apply SKY methods to short term analysis of PCa patient samples using methods we have refined in Phase 1. We believe that some deletions of chromosome arm 8p will be submicroscopic since they are apparent by molecular analysis such as LOH but by cytogenetic methods cannot be seen. We are therefore developing high resolution CGH microarrays as part of the second phase of this work to characterize the copy number of genomic probes such as PACs that map to this regions of interest 8p12, 8p22, and 8p23. In addition to chromosome 8 we will continue to study other chromosomal regions that emerge from our ongoing SKY analysis of patient samples.

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Princess Margaret Hospital
University Health Network

July 21, 1999

David L. Cooper
Editor-in-Chief
Nichols Institute/Quest Diagnostics Incorporated
San Juan Capistrano, CA

Dear Dr. Cooper,

Please find the enclosed original research manuscript:

Identification of a high frequency of chromosomal rearrangements in the centromeric regions of prostate cancer cell lines by sequential Giemsa-banding and spectral karyotyping

By Ben Beheshti and colleagues for publication in *Molecular Diagnosis*. This study is the first to apply sequential G-banding and spectral karyotyping (SKY) to search for common structural chromosomal aberrations in prostate cancer. A clustering of rearrangements was detected in centromeric locations in two prostate cancer cell lines (DU145 and PC-3). Both these lines were found to have karyotypes with a greater level of complexity than the LNCaP cell line, suggesting an underlying chromosomal instability associated with the cell lines derived from patients with more advanced disease.

There has been no duplicate publication or submission elsewhere of any part of this work. All authors have read and approved the manuscript.

I would also like you to consider having one of the color SKY figure elements as a cover photograph if this paper is found to be suitable for publication.

We hope this work is of interest to your readership and look forward to a speedy response.

Please contact me if you require further information.

Sincerely,

Jeremy Squire Ph.D.
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Identification of a high frequency of chromosomal rearrangements in the centromeric regions of prostate cancer cell lines by sequential Giemsa-banding and spectral karyotyping.

B. Beheshti[1,2], B.Sc., J. Karaskova[1], M.Sc., P.C. Park[1,2], Ph.D., J. Squire[1,2], Ph.D, and B.G. Beatty[1,2], Ph.D. Ontario Cancer Institute, Princess Margaret Hospital, University Health Network[1], and Department of Laboratory Medicine and Pathobiology, and Medical Biophysics, Faculty of Medicine, University of Toronto[2], Toronto, Ontario, Canada.

Key words: cytogenetics, fluorescence *in situ* hybridization, translocation breakpoint, centromere.

Running title: G-banding and SKY analysis of LNCaP, DU145, and PC-3.

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ABSTRACT

BACKGROUND: Currently, prostate cancer (CaP) cytogenetics is not well defined, largely due to technical difficulties in obtaining primary tumor metaphases.

METHODS AND RESULTS: We examined three CaP cell lines (LNCaP, DU145, PC-3) using sequential Giemsa-banding and spectral karyotyping (SKY) to search for a common structural aberration or translocation breakpoints. No consistent rearrangement common to all three cell lines was detected. A clustering of centromeric translocation breakpoints was detected in chromosomes 4, 5, 6, 8, 11, 12, 14, and 15 in DU145 and PC-3. Both these lines were found to have karyotypes with a greater level of complexity than LNCaP.

CONCLUSIONS: The large number of structural aberrations present in DU145 and PC-3 implicate an underlying chromosomal instability and subsequent accumulation of cytogenetic alterations that confer a selective growth advantage. The high frequency of centromeric rearrangements in these lines indicates a potential role for mitotic irregularities associated with the centromere in CaP tumorigenesis.

INTRODUCTION

Prostate cancer (CaP) is the leading cancer incidence and the second most common cause of cancer mortality in men in North America [1]. However, our understanding of the molecular genetic changes that underlie the progression of this disease remains at an early stage. Since it is well known that chromosomal translocations can lead to disruption of tumor suppressor gene function as well as activation of proto-oncogenes [2], identification of such rearrangements is a critical step towards understanding the development of this tumor. There are numerous examples in leukemias and soft tissue sarcomas where detailed cytogenetic analysis has identified consistent chromosomal aberrations leading to the isolation of causative genes [3, 4].

The cytogenetics of solid tumors has been hampered in comparison to hematological malignancies due to poor success in short-term culture, and inadequate representative metaphase spreads of good quality. Prostate cancer has been particularly problematic in this regard, since the tumor is slow-growing with a low mitotic index, and consequently there is a greater risk that normal stromal cell overgrowth will occur within a short duration of culture [5-7]. To circumvent some of these difficulties a variety of different tissue culture protocols have been implemented, including selection in favor of tumor cells and against normal cell overgrowth [8-12]. Using such procedures a number of consistent cytogenetic alterations have been identified generally affecting chromosomes 7, 8, 10, and Y [5, 13-15]. Nevertheless, no consistent structural chromosome aberrations have been identified in CaP and it remains conceivable that technical limitations on the quality of the cytogenetic preparations derived from primary tumor material have precluded identification of causative structural chromosomal alterations in this tumor.

In light of these difficulties, the detailed study of CaP cell lines has provided some insight into the progression of the disease and classical Giemsa-banding (G-banding) analysis of three of the commonly studied CaP cell lines, LNCaP, DU145, and PC-3 has provided useful information on the extent of cytogenetic change and karyotype evolution [16-19]. Cytogenetic analysis of LNCaP using standard G-banding methods revealed a relatively simple karyotype involving one

reciprocal and one nonreciprocal translocation, and three deletions [17]. The t(6;16)(p21;q22) translocation was recently shown to result in the production of a novel chimeric fusion transcript, Tpc-Hpr, that is thought to interfere with normal ribosomal function [20]. This translocation appears to be an isolated finding, as neither DU145 nor PC-3 has this rearrangement. However, both these lines have highly aberrant karyotypes in comparison to LNCaP and show many marker chromosomes and complex rearrangements with compound regions that cannot be identified by G-banding [18, 19]. Although the use of chromosome painting has helped in the identification of some of the complex marker chromosomes in these two cell lines, the origin(s) of many of these highly abnormal chromosomes remains unknown [21].

In order to more accurately define the karyotypes of these three cell lines, we have used the new technique of spectral karyotyping (SKY) in combination with G-banding. SKY is a "24-color" fluorescence *in situ* hybridization (FISH) approach that uniquely identifies each chromosome based on its specific spectral color composition [22], and the technique allows for the unambiguous identification of individual chromosome fragments involved in complex chromosomal rearrangements and marker chromosomes. By analyzing SKY results in conjunction with the findings from conventional G-banding using the same metaphase spread, it is possible to identify individual regions of specific chromosomes and accurately define all structural rearrangements present.

In this study we have applied sequential G-banding and SKY to the three CaP cell lines LNCaP, DU145 and PC-3 in order to: (1) search for all previously unidentified structural chromosomal rearrangements in each cell line; (2) determine if there are any consistent rearrangements, or cryptic or 'masked' chromosomal changes common to all three cell lines; and (3) fully characterize the more complex chromosomal rearrangements present in DU145 and PC-3.

MATERIALS AND METHODS

Tissue Culture and Cytogenetic Preparations

LNCaP (CRL-1740), DU145 (HTB-81), and PC-3 (CRL-1435) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). LNCaP, an androgen-dependent cell line originating from a lymph node metastasis [16, 23], was grown in RPMI 1640 with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. DU145, an androgen-independent cell line obtained from a metastasis to the bone [18], was grown in F15K Minimum essential medium with 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. PC-3, also an androgen-independent cell line and originated from a brain metastasis [19], was grown in Ham's F12K with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum.

Cytogenetic preparations of LNCaP (passage 23), DU145 (passage 83), and PC-3 (passage 38) were made according to standard protocols [24] using colcemid and KCl hypotonic treatment. The slides were karyotyped following a standard G-banding protocol [24], and images of ten metaphases in which there was minimal chromosome overlap, long chromosome length, little or no cytoplasm, and high banding resolution were selected for detailed analysis. Microscope co-ordinates of all digitized G-banded preparations were recorded, so that the metaphase cells analyzed by G-banding could be analyzed concurrently by SKY methods.

Spectral Karyotyping (SKY)

The SKY™ KIT probe cocktail from Applied Spectral Imaging (ASI, Carlsbad, CA) was hybridized to metaphase spreads from each CaP cell line according to standard protocols [22, 25, 26] and the manufacturer's instructions (ASI, Carlsbad, CA). Briefly, after destaining the G-banded slides with methanol for 10 minutes, the slides were rehydrated in a descending ethyl alcohol series (100%, 90%, 70%), and fixed with 1% formaldehyde in 50mM $MgCl_2$/phosphate buffer solution for 10 minutes. The slides were then dehydrated using an ascending ethyl alcohol

series, and denatured for 30-45 seconds in 70% formamide/2XSSC at 75°C. The SKY probe was denatured for 10 minutes at 75°C, reannealed at 37°C for 1 hour, placed on the slide and covered with a glass coverslip. The coverslip was sealed with rubber cement and the slides placed in a damp container in a 37°C incubator. After hybridizing overnight, the post-hybridization washes were performed per manufacturer's instructions (ASI, Carlsbad, CA).

The metaphase images were captured using an SD 200 spectral bio-imaging system (ASI Ltd., MigdalHaemek, Israel) attached to a Zeiss microscope (Axioplan 2) and stored on a SKY image-capture workstation. The images were analyzed using the SKYView software version 1.2 (ASI, Carlsbad, CA), which resolves individual fluorochrome spectra by Fourier spectroscopy and distinguishes the spectral signatures for each chromosome to provide a unique pseudocolour for each chromosome (classified image). G-banding and SKY analyses were performed sequentially on each of the three cell lines with the same ten metaphase images captured for G-banding also analyzed by SKY. Because of the presence of nonclonal changes in DU145 and PC-3, composite karyotype descriptions were made for these two cell lines.

The determination of the position of translocation breakpoints was performed by aligning the G-banding pattern for each rearranged chromosome with its respective SKY pseudocolor classified image, and mapping each translocation boundary with respect to the associated G-banded chromosomal interval and the ISCN designation [27] for the band locations where breakage and rearrangement has occurred.

RESULTS

Sequential G-banding and SKY analysis of LNCaP cells on a metaphase-by-metaphase basis confirmed the bimodal diploid and tetraploid chromosome number [17]. Overall, LNCaP demonstrated a consistent karyotype, with few nonclonal changes (incidental gains/losses and/or structural rearrangements not contributing to the karyotype) per metaphase (Table 1). Six of the seven previously reported marker chromosomes [17] were confirmed by G-banding and SKY analyses in 10/10 metaphases (Figure 1). Marker 7 (an interstitial deletion of 13q21.1), was detected in 9/10 metaphases but was absent in the metaphase shown in Figure 1. The level of resolution afforded by the current sensitivity of the SKY system enabled identification of a cryptic or 'hidden' novel rearrangement in LNCaP. Markers 3 and 6, previously identified by G-banding to be involved in a nonreciprocal translocation of a fragment of 6p onto 16q [17], were instead found by SKY to be involved in a reciprocal t(6;16). When normalized to a diploid chromosome number, LNCaP cells were found to have 9 structural aberrations per metaphase. For example: the reciprocal t(1;15)x2 counted as four aberrations, the der(6)t(4;6)x2 as two aberrations, and the del(2) as one aberration; numerical changes such as the loss of chromosome 2 were not included in the count. G-banding and SKY analysis of LNCaP metaphase cells revealed few structural aberrations per metaphase, indicating that the karyotype was relatively simple.

As previously reported [18, 21], DU145 was observed to have a hypotriploid chromosome number with more complex karyotypic changes than LNCaP, showing approximately 18 aberrations per diploid cell (Figure 2). Chromosomal loss in DU145 was more common than gain, with losses of whole chromosomes 2, 3, 4, 13, 16, 19, 20, 21, 22, and X, and partial losses of 5q, 9p and 11q; and gains of chromosome 18 and derivative chromosomes 5 and 9 (Table 1). Structural chromosomal changes of interest were the t(5;21) and t(4;6) translocations that were not detected by G-banding analyses, but were easily identified by SKY. Other translocations, such as the t(1;4), t(Y;20), t(2;13), t(6;16) and t(9;11), were recognized as abnormal derivative chromosomes 1, Y, 2, 6 and 9 by G-banding analysis, but required SKY analysis for identification of the involved partner

chromosomes (Table 1). The previously unidentified minute chromosomes observed by Stone et al. [18] were determined by SKY analysis to be derived from chromosome 5. The sequential analysis of DU145 by G-banding and SKY allowed the identification of 27 structural breakpoints of which 14 involved centromeric or pericentromeric regions. More than half of the chromosomes in the DU145 genome showed rearrangements involving centromeric breaks. DU145 demonstrated more nonclonal changes per metaphase than either LNCaP or PC-3.

PC-3 cells were also observed to be hypotriploid and demonstrated more karyotypic abnormalities than either LNCaP or DU145 cells, with approximately 34 aberrations per diploid cell. Almost every chromosome in this cell line had either structural or numerical abnormalities (Figure 3) with chromosomal loss being more prevalent than gain. PC-3 exhibited losses of whole chromosomes 3, 5, 8, 9, 10, 15, 16, 17, 19, and 22, and partial loss of chromosomes 6q, 8p, and 17p. In addition, whole chromosomal gains of 1, 7, 11, 14, 20, and 21 were observed, and an additional gain of chromosome 14 was observed in 4/10 metaphases (Table 1). Seven complex rearrangements involving more than 2 chromosome partners were characterized in this cell line. Sequential G-banding and SKY analysis of PC-3 allowed the identification of 37 structural breakpoints of which 8 involved centromeric or pericentromeric regions. Many of the structural rearrangements were paired, suggesting that these changes occurred in a diploid progenitor that subsequently underwent tetraploidization. The isochromosome 5p, previously reported by Bernadino et al. [21], was also identified by SKY in both DU145 and PC-3.

In both DU145 and PC-3, marker chromosomes which had been partially characterized by classical banding and chromosome painting approaches were more fully characterized by sequential G-banding and SKY. Listed in Table 2 are several examples of marker chromosomes whose identities previously reported by Bernardino et al. [21] have been further defined by sequential G-banding and SKY analyses.

The composite G-banding and SKY karyotype results for the three CaP cell lines shown in Table 1 demonstrate an increasing complexity of chromosomal aberrations, with LNCaP having the simplest pattern of chromosomal change, followed by DU145 with intermediate complexity, and

PC-3 as the more complex line. While no consistent rearrangement or common chromosomal aberration was detected using the increased sensitivity afforded by SKY, examination of DU145 and PC-3 revealed eight chromosomal rearrangements involving breakage within the centromeric regions of chromosomes 4, 5, 6, 8, 11, 12, 14, and 15 (Figure 4). Furthermore, DU145 was found to have involvement of eight additional centromeric or pericentromeric rearrangements on chromosomes 1, 2, 7, 10, 16, 19, 20, and 21; PC-3 had only one additional involvement on chromosome X.

DISCUSSION

Obtaining a detailed characterization of chromosomal abnormalities in solid tumors by classical cytogenetics has been limited by difficulties in both culturing fresh tumor tissue and in obtaining good quality representative banded metaphase preparations. The use of tumor cell lines has provided an alternative resource for studying cytogenetic changes in greater depth, and the recent development of SKY has significantly enhanced the ability to detect and comprehensively identify the structural aberrations present in any cell line [28]. However, SKY analysis as a single method of chromosome identification has significant limitations. For example, the current SKY probe kit does not permit detection of intrachromosomal dosage changes or interstitial structural rearrangements. In addition, SKY classification does not provide information on the region of the abnormal chromosome involved in the rearrangement. We have therefore used a sequential approach of G-banding followed by SKY to examine the identity of all chromosomal aberrations present in the three CaP cell lines, LNCaP, DU145 and PC-3. Similar sequential methods were recently reported to identify the origins an unusual marker chromosome in a leukemia [29].

The advantages of the sequential approach of G-banding and SKY are evident in genomes demonstrating increased karyotypic complexity, such as DU145 and PC-3. G-banding data for both these cell lines [18] was unable to fully characterize the observed chromosomal aberrations. The use of pair-wise combinations of chromosome paints provided more information on the chromosomes involved in rearrangements [21]. For example, while Stone et al. identified a marker Y chromosome by G-banding, the partner chromosome was unidentifiable by this method [18]. Bernardino et al. [21] used pair-wise combinations of chromosome-painting FISH experiments to resolve the identity of this marker chromosome as a der(Y)t(Y;20)(q12;?). Despite these advantages, karyotyping by pair-wise chromosome painting is cumbersome and limited by the number of potential combinations of chromosomal rearrangements found in derivative chromosomes. Combined G-banding and SKY has overcome these limitations and permitted

further characterization of novel rearrangements and more precise definition of previous rearrangements in DU145 and PC-3 (Table 2).

The chromosomal stability of the karyotypes present in each cell line is also a consideration when comparing cytogenetic findings ascertained using different sources of the same cell line and at different cell passage number. In our analysis, sequential G-banding and SKY revealed a cryptic novel translocation of a small fragment of 16q onto 6p, but did not reveal any additional chromosomal changes in LNCaP in comparison to previous G-banding results [17]. However, a study by Ford et al. using whole-chromosome paints [30] detected the nonreciprocal translocation of 10q24 material to two sites on chromosome 5q forming a derivative chromosome 5 that was not present in our analysis. Similarly, a recent SKY analysis of LNCaP cells reviewed by Brothman et al. [31] demonstrated additional chromosomal rearrangements, such as t(15;22) and t(3;11), also not observed in our analysis. Whether these rearrangements in LNCaP are representative clonal changes is unclear. Previous studies have shown that the karyotype of DU145 also varies as a function of passage number. Both Stone et al. and Bernardino et al. found that the DU145 karyotype was stable through 90 passages, and at passage 73 the cells had a near-triploid chromosome number with extensive chromosomal rearrangements [18, 21]. By passage 153, however, DU145 was found to have a near-tetraploid karyotype with an increased number of rearrangements [18, 21]. The karyotype for DU145 (passage 83) reported herein is comparable with that reported by Stone et al. and Bernardino et al. below passage 90 [18, 21]. In contrast to DU145, PC-3 is believed to be a karyotypically stable cell line [19]. This is supported by the study of Camby et al. [32] that showed PC-3 to be more hormone-sensitive and to maintain a higher degree of differentiation than DU145. Kaighn et al. described PC-3 as 100% aneuploid with complete losses of chromosomes 1, 2, 3, 5, 15, and Y; and the presence of at least ten marker chromosomes per metaphase spread [19], a finding confirmed by the present study. The prevalence of chromosomal losses over gains seen in both DU145 and PC-3 is supported by recent comparative genomic hybridization (CGH) findings [33].

Our results for the CaP cell lines showed that in terms of the karyotypic complexity of rearrangements, LNCaP < DU145 < PC-3, with approximately 9, 18, and 34 structural aberrations per diploid cell, respectively (Table 1). This finding is in agreement with the suggestion by Nupponen et al. that DU145 and PC-3 represent the more advanced, androgen-independent CaP disease state while LNCaP resembles more closely primary CaP disease [33]. This would support the concept that the stepwise progression to a more advanced disease state, as modeled by DU145 and PC-3, involves an accumulation of chromosomal alterations that may confer selective growth advantages.

Sequential G-banding and SKY analyses demonstrated that there was no common chromosomal rearrangement or common translocation breakpoint present in all three CaP cell lines. When comparing breakpoint regions of DU145 and PC-3, the most common shared feature was involvement of the centromeric regions of chromosomes 4, 5, 6, 8, 11, 12, 14, and 15 in structural chromosomal aberrations. In contrast, LNCaP was observed to have only one centromeric rearrangement on chromosome 10. The high involvement of the centromeric regions in DU145 and PC-3 is of interest because the centromere plays an essential role in maintaining diploidy [34]. The greater frequency of aberrations at centromeric and pericentromeric regions in DU145 than PC-3 may be of importance given the increased instability observed in DU145 through passaging [18, 21]. Only monocentric chromosomes were observed in all three cell lines suggesting orderly chromosome separation, which is not seen in cells containing ring, dicentric and multicentric chromosome structures [34]. Normally the centromere is the last chromosomal segment that is replicated in monocentric mammalian chromosomes during cell division [35]; however, premature centromere separation could lead to the type of aneuploidy [34] observed in DU145 and PC-3. The significant involvement of centromeric breakpoints may reflect the high degree of chromosomal misdivision and sister-chromatid exchange, or increased instability of the pericentromeric regions during mitosis [34, 36]. There is an increasing interest in understanding the role of the kinetochore in normal and abnormal mitosis [37, 38] and its relationship to the acquisition of centromeric aberrations and aneusomies in cancer cells [39, 40].

While the use of tumor cell lines has provided an alternative resource for studying cytogenetic changes in carcinomas that ordinarily would present difficulties in tissue culture, the question is raised as to whether the cytogenetics remain representative of primary tumors. Given the slow onset pathology of CaP, however, it may be surmised that the initiating event(s) may not be a single genetic alteration, but instead due to aberrations in cell division. In this regard, the accumulation of multiple genetic aberrations during CaP progression may be downstream effects which confer selective growth advantages. The observed alterations at the centromeric regions support this view and suggest that amidst the other chromosomal aberrations within each cell line, the initial tumorigenic events are not lost in the cell lines studied.

In summary, sequential G-banding and SKY is an effective FISH-based whole-genome screening technique that significantly improves the ability to identify cryptic and complex chromosomal rearrangements in tumor cells. Using this approach we have confirmed and more precisely defined the karyotypes of three CaP cell lines, identifying a cryptic novel rearrangement in LNCaP and resolving previously unknown marker chromosomes and complex rearrangements in the more complicated DU145 and PC-3 genomes. No consistent translocation breakpoint, suggestive of a common structural rearrangement in all three cell lines was observed; however, centromeric breakpoints were demonstrated to be the most frequent shared feature between DU145 and PC-3. Our results imply that karyotypically, LNCaP may be less advanced than DU145 and PC-3. This observation is in agreement with the multistep model of accumulated hits in CaP tumorigenesis and suggests an increasing importance in understanding the role of the centromere in CaP tumorigenesis.

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FIGURE LEGENDS

Figure 1. G-banding and SKY composite karyogram of LNCaP (passage 23). Giemsa-banded metaphase (top left), spectral metaphase (top middle), pseudocolour classification (top right). There are 87 chromosomes in the metaphase spread. The karyogram (bottom) depicts each chromosome by aligning its G-banded (left chromosome) and classified (right chromosome) representations.

Figure 2. G-banding and SKY composite karyogram of DU145 (passage 83). There are 60 chromosomes in the metaphase spread.

Figure 3. G-banding and SKY composite karyogram of PC-3 (passage 38). There are 62 chromosomes in the metaphase spread.

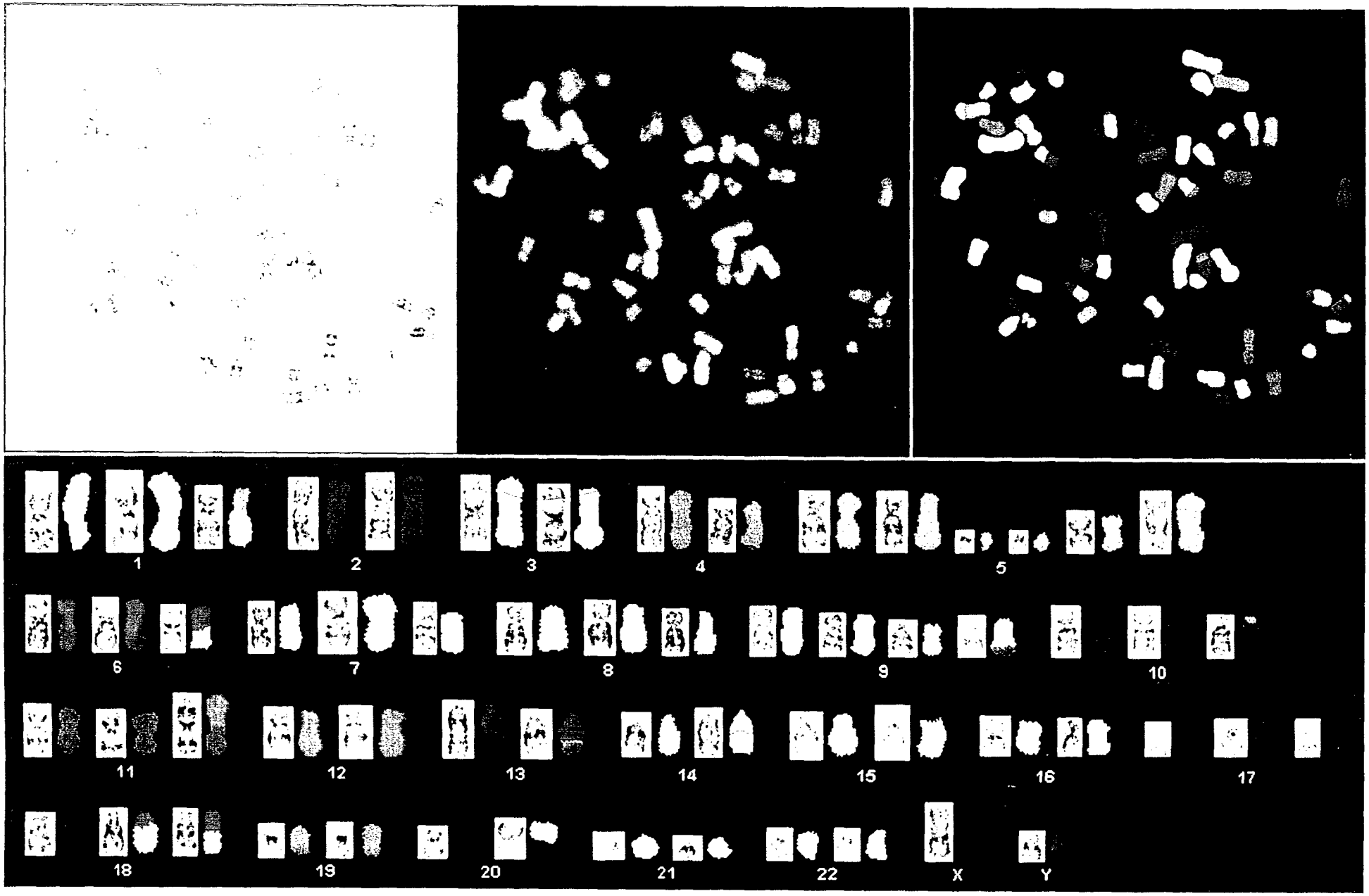
Figure 4. Breakpoint analysis of the three CaP cell lines. Breakpoints found in the CaP cell lines (LNCaP—red; DU145—blue; PC-3—green) are designated as circles to the right of each chromosome ideogram in the centre of the chromosomal interval where the breakpoint occurs. Clustering of centromeric and pericentromeric breakpoints in DU145 and PC-3 are indicated as bars to the left of the ideograms (cyan).

Cell line	Chromosomal Rearrangements	Structural Aberrations per Diploid Cell
LNCaP	86~90,XXYY,t(1;15)(p22;q24)x2,-2,del(2)(p13~23),der(4)t(4;6)(q21;q?15)t(6;10)(q?25;q11)x2, der(6)t(4;6)(q25;q15)x2,t(6;16)(p21.1;q22)x2,del(10)(q24)x2,del(13)(q21.1),[10]	18/2 = 9
DU145	57~62<3n>,X,-X,der(Y)t(Y;20)(q12;?p11)[10],der(1;4)(q10;p10)[9],-2[10],-3[10],-4[9], der(4)t(4;6)(q31;?)[9],i(5)(p10)[10],+der(5)del(5)(p?13)del(5)(q?11)x2[8], +der(5)t(5;21)(p13;q11.2)[10],der(6;16)(p10;q10)[9],der(7;8)(p10;q10)[7],del(9)(p21)[10], +der(9)del(9)(p13)t(9;11)(q22;?)[8],der(10;19)(q10;?p10)[9],del(11)(q23)[10], der(11;12)(q10;q10)[9],-13[10],der(13)t(2;13)(?p11;q33)[10],der(13)t(11;13)(?q23;q33)[10], der(14)t(3;14)(q21;q31)[8],ider(14)(q10)t(3;14)(q21;q31)[2],der(15;20)(q10;q10)x2[10], -16[10],+18[8],der(18)t(14;18)(q13;q21)x2[10],-19[10],-20[10],-21[10],-22[10],[cp10]	27*(2/3) = 18
PC-3	59~64,XX,-Y,+1[7],der(1)t(1;2)(q22;?)t(1;12)(p31;?)t(8;12)(q13;?)[8], der(1)t(1;15)(p22;q15)t(1;2)(q25;?p21)[9],der(2)t(2;15)(p24;q22)t(15;17)(q11;q12)[10], der(2)t(2;8)(p24;q13)x2[10],-3[10],der(3)t(3;10)(q13;?)x2[10],der(4;6)(q10;p10)[9], der(4)t(4;10)(q21;?)x2[10],der(4;12)(q10;q10)[10],-5[10],i(5)(p10)[9],del(6)(q25~26)[10], +7[9],-8[9],del(8)(p21)[10],der(8)t(X;8)(q10;q10)[5], der(8)t(8;15)(q10;q10)t(15;?17)(q26;?)t(3,?17)(q25;?)[3],-9[9],-10[9], der(10)t(3;10)(p14.1;q21)t(4;10)(?;q25)t(4;10)(?;q21)t(4;10)(?;p12)t(4;10)(?;?)t(3;10)(q13.3;?)x2[10], +11[7],der(11)t(2;11)(?;p11)t(2;19)(?;?)t(5;19)(q13;?)[10],der(11;14)(q10;q10)[3], der(12)t(8;12)(q13;q24.3)x2[10],+14[7],+14[4],der(14)t(X;14)(?p22.1;q32)x2[6], der(14)dup(14)(?)t(14;15)(p12;?)t(15;17)(?;?)t(3;17);(q25;?)[2],-15[10], der(15)t(5;15)(q13;p13)[10],der(15)t(15;17)(?;?)t(15;17)(?;q21)t(3;17)(q25;?)[8],-16[10], -17[9],del(17)(p11.2)[10],-19[8],+20[6],+21[10],-22[10],[cp10]	51*(2/3) = 34

Table 1. Karyotype description of LNCaP (passage 23), DU145 (passage 83), and PC-3 (passage 38) by sequential G-banding and SKY, according to the ISCN convention [27]. LNCaP has a clonal karyotype (ten metaphases). In the composite karyotype descriptions for DU145 and PC-3, numbers in brackets refer to the frequency of occurrence of the directly preceding structural/numerical change (out of ten metaphases).

Cell line	Chromosome Painting Results	Identity by G-banding and SKY
DU145	• add(13)(q33)	• der(13)t(2;13)(?p11;q33)
	• add(13)(q33)	• der(13)t(11;13)(?q23;q33)
	• add(5)(p13)	• der(5)t(5;21)(p13;q11.2)
	• 1-5 markers	• eg.: der(5)del(5)(p?13)del(5)(q?11)x2; der(14)t(3;14)(q21;q31)
PC-3	• add(14)(q32)	• der(14)t(X;14)(?p22.1;q32)
	• der(15)t(5;?;15)(q14;?;p12)	• der(15)t(5;15)(q13;p13)
	• der(11)t(5;10;11)(q14;?;p11)	• der(11)t(5;2;11;19)(q13;?;p11;?)
	• hsr(10)(1;3;10)	• der(10)t(3;10;4;10)
	• der(2)t(2;?;8)(p25;?;q21)x2	• der(2)t(2;8)(p24;q13)x2
	• add(2)(p25)	• der(2)t(2;15;17)
	• 2-5 markers	• eg.: der(8)t(X;8)(q10;q10); der(1)t(2;1;12;8)

Table 2. Examples of chromosome rearrangements that were previously identified by chromosome painting [21] experiments (left), and probable identities found by G-banding and SKY analysis (right). See Table 1 for correct ISCN classifications of listed results.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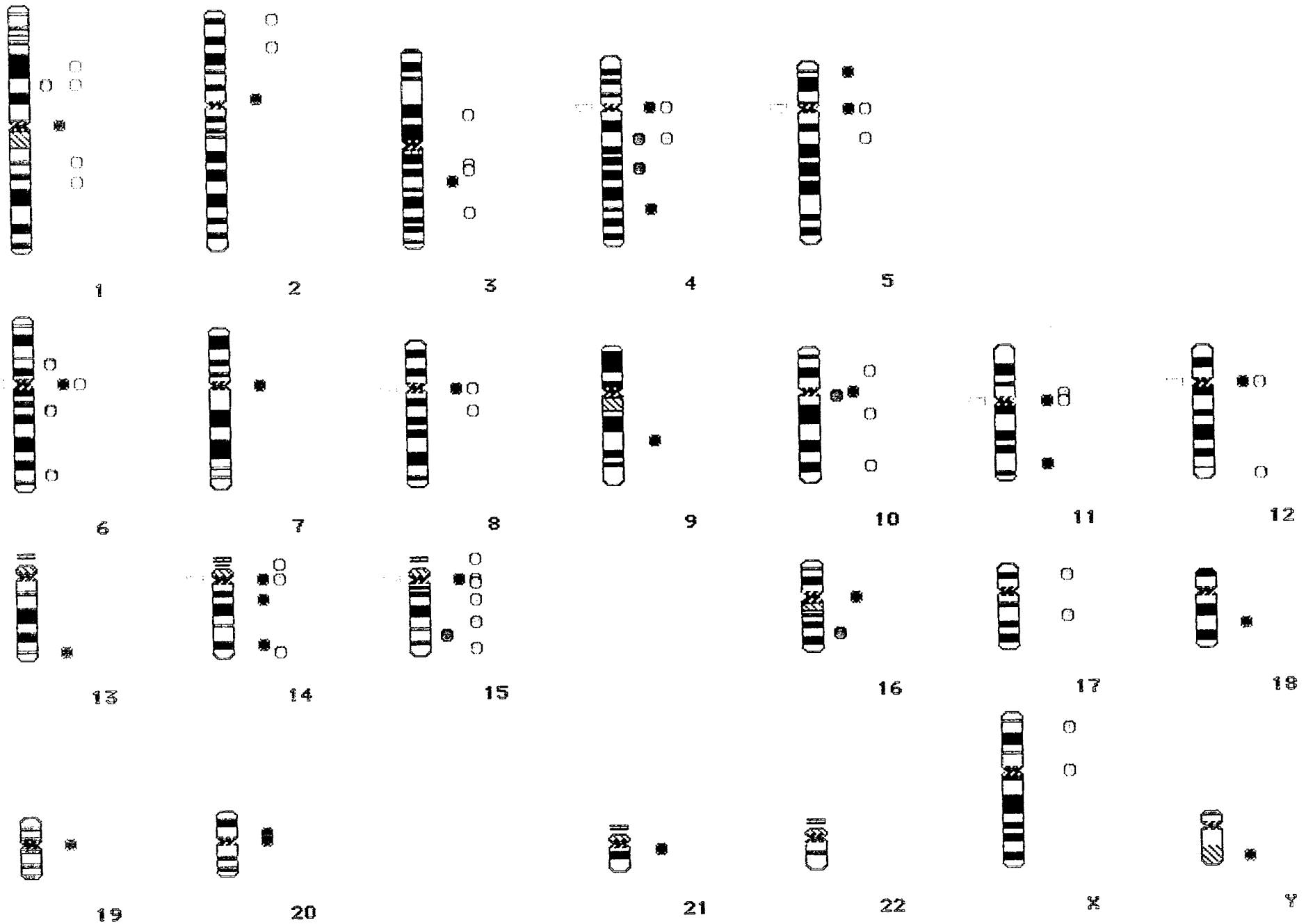
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Genetic Characterization of Immortalized Prostate Epithelial Cell Cultures: Evidence for Structural Rearrangements of Chromosome 8 and i(8q) Chromosome Formation in Malignant-Derived Cells

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ABSTRACT

We have utilized a combination of conventional and spectral karyotyping (SKY) techniques and allelotype analysis to assess numerical and structural chromosomal alterations in one normal- and three malignant-derived prostate epithelial cell lines immortalized with the E6 and E7 transforming genes of human papilloma virus (HPV) 16. These studies revealed trisomy for chromosome 20 and rearrangements involving chromosomes 3, 8, 10, 18, 19, 20 or 21. In addition, all four cell lines exhibited extensive duplications or translocations involving the 11q13q22,q23 chromosomal region. Interestingly, the spectral karyotyping data revealed that the loss of 8p sequences detected by allelotyping in two of the cell lines was directly due to i(8q) chromosome formation and/or other structural alterations of chromosome 8. This provides intriguing evidence that 8p loss in human prostate tumors may, in some cases, result from complex structural rearrangements involving chromosome 8. Furthermore, this data provides direct evidence that such complex structural rearrangements sometimes includes i(8q) chromosome formation.

INTRODUCTION

It is likely that many genetic and epigenetic events are involved in prostate tumorigenesis. In particular, several cytogenetic and molecular studies from our laboratory and others have suggested that deletion or rearrangement of sequences that map to the short arm of chromosome 8 (8p) may be critically permissive for tumorigenesis in the prostate gland (1-7). Deletion of 8p sequences is observed at comparable frequencies in low- and high-grade tumors, as well as in localized and invasive/regionally metastatic prostate cancers (3,5,7). Moreover, the frequency of 8p loss is almost equivalent in prostate tumors and prostatic

intraepithelial neoplasia (PIN), a putative premalignant lesion of the prostate (6,**8**). Taken together, these data suggest that 8p losses are frequent events during the initiation or early promotion of prostate tumorigenesis.

Other studies have also reported loss of 8p concurrent with gain of the long arm of chromosome 8 (8q) sequences in advanced prostatic cancer (**9**-12). This combination of events occurring on the same chromosome - loss of 8p sequences and gain of 8q sequences - suggests formation of i(8q) chromosomes in advanced prostate tumors. However, no direct evidence for the existence of i(8q) chromosomes in prostate tumors has been detected due to the inability of interphase FISH techniques used with clinical specimens to accurately and precisely identify these chromosomes (12). Therefore, we have utilized a combination of conventional and spectral karyotyping techniques and allelotype analysis to assess numerical and structural alterations of chromosome 8 in one normal- and three malignant-derived immortalized prostate epithelial cell lines. The specific objective of these studies was to determine whether losses of 8p sequences previously reported for two of the cell lines, 1532T and 1542T (13), were directly due to i(8q) chromosome formation and/or other structural alterations of chromosome 8.

MATERIALS AND METHODS

G-Banding and Kayotypic Analysis

Chromosome counts, ploidy distributions and GTG-banded karyotypes were prepared as previously described (14). Briefly, exponentially growing cultures were treated with 0.04 ug/ml Colcemid for 1-2 hours, trypsinized, treated with 0.0375 M KCl for 9 minutes, then fixed in 3:1 methanol:glacial acetic acid. The resulting cell nuclei were pelleted by centrifugation, dropped onto cold, wet slides, then air dried and stained using a 4% Giemsa solution. Chromosomes were examined and counted to establish ploidy distribution and constitutional alterations. Specific numerical and structural chromosomal alterations were established after the slides were aged at 60°C on a slide warmer for 18 hours, immersed in 0.025% trypsin for 11 seconds, stained with 4% Gurr-Giemsa solution for 11 minutes, washed in buffer, then air-dried and mounted in permount. Well-banded metaphase spreads were photographed at 800X magnification with Technical Pan Film 2415 (Kodak) and printed on Rapidoprint FP 1-2 (Agfa-Gevaert), or studied on the AKSII image analysis system. Nine (1535N, 1532T) or ten (1535T, 1542T) karyotypes were prepared and examined for each cell line.

SKY Analysis

Spectral karyotyping (SKY) analysis was carried out on previously G-banded slides. Images were captured and the microscope coordinates were noted. Residual oil was removed with xylenes followed by destining with methanol. The slides were then rehydrated in a descending ethanol series and fixed in a 1% formalin solution followed by a 1X PBD

(phosphate-buffered detergent) wash. Slides were dehydrated and denatured at 75C in 70% formamide/2X SSC (saline sodium citrate) for 40 seconds followed by a final dehydration. The SKY paints (Applied Spectral Imaging, Carlsbad CA) were allowed to hybridize for 24 hours to the denatured slides. Post hybridization washes and hapten detections were carried out using established techniques (15) and according to manufacturer's instructions. Ten metaphase images were captured using Applied Spectral Imaging's software v1.2 and analyzed using SKYVIEW v3.1.

Allelotyping

Cells were trypsinized and DNA was purified using the Oncor (Gaithersburg, MD) non-organic DNA extraction kit according to manufacturer's protocols. PCR reactions were accomplished as previously described (7). The loci examined by PCR spanned 8p (12 loci) or localized to 8q12 (2 loci), and contained highly polymorphic microsatellite repeats. The linkage order of these markers has been reported as pter - D8S504 - D8S277 - D8S549 - D8S261 - *NEFL* - D8S540 - D8S513 - D8S535 - D8S505 - D8S87 - D8S1121 - D8S255 - D8S531 - D8S519 - qter (see Table I). Primer sequences, additional linkage and contig information, and genetic mapping information were obtained from public databases maintained by the Center for Genome Research at the Whitehead Institute for Biomedical Research (http://www-genome.wi.mit.edu/), and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), as accessed through the Internet.

RESULTS

Cytogenetic Analysis

Metaphase analysis showed that the four prostate-derived cell lines were pseudo-diploid, with modal numbers ranging from 43-49 chromosomes/cell. The karyotypes of each cell line are described below and in Table II.

1532T. Ten karyotypes were analyzed from passage 44 of this cell line using spectral karyotyping techniques. The consensus karyotype was 44~47,XY,i(8)(q10),+20. Eight cells also demonstrated duplication of the (q13q23) region of chromosome 11, and five cells demonstrated a duplication of the p11.2p13 region of chromosome 11 and insertion into the q21 region of chromosome 17. Figure 1 shows a representative karyotype for this cell line.

1535T. Ten karyotypes were analyzed from passage 12 of this cell line using spectral karyotyping techniques. The consensus karyotype was 46,XY,der(11)?qdp(q13q23)t(11;20)(q23;q11)der(20)t(11;20)(q13;q13.3)qdp(11)(q13q23) or hsr(11), with four cells also demonstrating der(3)t(3;11)(p21;q13),del(18)(q21). Figure 2 shows a representative karyotype for this cell line.

1542T. Ten karyotypes were analyzed from passage 44 of this cell line using spectral karyotyping techniques. The consensus karyotype was 46,XY,der(8;20)(q10;p10),der(11)qdp(q13q23)t(11;20)(q23;q11). In addition, 2 cells also demonstrated an i(8)(q10); two cells demonstrated these changes as well as

der(22)t(11;22)(q14;p11)t(11;20)(q23;q11.2), and two cells were characterized by these accumulated changes except the der(8;20) was absent and a der(8;21)(p10;q10) was apparent instead. Figure 3 shows a representative karyotype for this cell line.

1535N. Nine karyotypes were analyzed from passage 13 of this cell line using G-banding techniques. The consensus karyotype was 45-48, XY, der (18), with eight cells also demonstrating an add(19)(q13) chromosome, and four cells demonstrating a complex derivative of chromosome 11 involving t(10,11)(qter?q21).q24?q13::q22>pter). Three cells also displayed a deletion of chromosome 10 involving band q21. Figure 4 shows a representative karyotype for this cell line.

Interestingly, duplications or translocations involving 11q13q22,q23 were observed in all four cell lines.

Allelotyping

The three tumor-derived cell lines were allelotyped at 14 chromosome 8 loci, 12 spanning 8p, and 2 mapping to the pericentromeric region of 8q. Table I summarizes this data, with gray-shaded areas indicating extended regions of homozygosity defined as the observation of three or more adjacent homozygous loci. As shown in Table I, the 1532T cell line was homozygous for all loci examined, consistent with the cytogenetic data revealing one normal chromosome 8 and one i(8)(q10) chromosome in these cells (Fig. 5). The 1542T cell line demonstrated one allele for all 8p loci, but two alleles for each of the pericentromeric 8q loci, D8S531 and D8S519. This data was also consistent with the cytogenetic findings for

one normal chromosome 8 accompanied by any of three different structural alterations of chromosome 8 - i(8)(q10); der(8,20)(q10;p10) and der(8;21)(p10;q10) - in these cells (Fig. 5). In contrast, the 1535T cell line demonstrated two alleles for 9/12 8p, and both 8q, loci examined, with no evidence for extended regions of homozygosity by allelotyping. These results were consistent with the spectral karyotyping data, which did not reveal clonal numerical or structural alterations of chromosomes 8 in these cells.

DISCUSSION

Cell lines provide a unique resource for the investigation of numerical and structural chromosomal alterations present in the tissues from which they were derived. However, the cell lines studied most intensively by prostate cancer investigators - PC3, DU145, LNCaP and TSU-Pr1 - were all established from metastatic lesions. As such, it is unlikely that these cell lines accurately recapitulate the genetic composition of primary prostate tumors. Unfortunately, prostate tissue, whether normal or malignant, survives only short term in culture, and rarely immortalizes spontaneously. The use of viral transforming proteins to immortalize normal and malignant prostate tissues has allowed the continual propagation of normal and malignant-derived cells *in vitro* (13). The cell lines examined in the present study were created by Bright et al. through the transduction, and subsequent immortalization, of normal and malignant prostatic tissues with the E6 and E7 transforming genes of human papilloma virus (HPV) 16 (13).

The cell lines demonstrated several numerical and structural chromosomal alterations, including trisomy for chromosome 20 (1532T cells) and rearrangements involving 3p (1535T

cells), chromosomes 10, 18, 19 (1535N cells), 20 or 21 (1542T cells). All of these alterations have been reported for epithelial cells from diverse tissue types, including uroepithelial and prostate, immortalized through transduction with all or part of the HPV 16 or 18 genomes (16-19). In addition, all four cell lines exhibited extensive duplications or translocations involving the 11q13q22,q23 chromosomal region. 11q+ alterations have been reported in cells immortalized with the HPV 16 or 18 genomes (16,17), and the 11q23 region has been classified both as a fragile site and possible viral modification site (20,21). It appears that the 11q+ alteration observed in the cell lines examined comprises the common chromosomal aberration directly due to immortalization with the E6 and E7 genes of HPV 16.

The four cell lines examined in the present study were partially allelotyped by Bright et al., who reported loss for a limited number of markers mapping to 8p in the 1532T and 1542T, but not the 1535N or 1535T, cell lines (13). Interestingly, the 8p loss pattern observed in the tumor tissues and resulting immortalized cell lines was concordant for the 1542T, but not 1532T or 1535T, cell lines. We have confirmed these results for the cell lines, and report a more precise allelotyping, with 12 markers spanning 8p and 2 markers pericentromeric to 8q (Table I). Complete loss of one copy of 8p in the 1532T and 1542T cell lines was observed, with loss extending pericentromerically into 8q in 1532T cells. These findings are remarkably similar to those reported by others describing reduction to homozygosity for all or part of 8p in prostate tumor tissues (1-10). Moreover, conventional G-banding and spectral karyotyping data revealed that loss of 8p sequences in the 1532T and 1542T cell lines was associated with complex structural alterations of chromosome 8 (Fig. 5). The 1532T cells exhibited an i(8q) chromosome in all 10 metaphases examined by spectral karyotyping. The 1542T cells also

demonstrated an i(8q) chromosome, as well rearrangement of chromosome 8 material with either chromosome 20 or 21. The karyotyping data provides intriguing evidence that 8p loss in prostate tumors may not result from simple deletion of all or part of the short arm, as has been previously inferred from allelotyping data (1-8). Rather, 8p loss may, in fact, result from complex structural rearrangements of chromosome 8, often resulting in gain of 8q material, which occurs during tumorigenesis. Moreover, the data reported here provides direct evidence that such complex structural rearrangements sometimes includes i(8q) chromosome formation.

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FIGURE LEGENDS

Figure 1. Spectral Karyotype Composite of the 1532T Cell Line. Upper Panel: G-banded preparation of metaphase chromosomes from 1532T cells (left), hybridized to SKY paints (middle), and after pseudo-color application (right), as described in the text. Lower Panel: Composite karyotype showing G-banded and pseudo-colored chromosomes. Numerical and structural chromosomal alterations are described in the text.

Figure 2. Spectral Karyotype Composite of the 15325T Cell Line. Upper Panel: G-banded preparation of metaphase chromosomes from 1535T cells (left), hybridized to SKY paints (middle), and after pseudo-color application (right), as described in the text. Lower Panel: Composite karyotype showing G-banded and pseudo-colored chromosomes. Numerical and structural chromosomal alterations are described in the text.

Figure 3. Spectral Karyotype Composite of the 1542T Cell Line. Upper Panel: G-banded preparation of metaphase chromosomes from 1542T cells (left), hybridized to SKY paints (middle), and after pseudo-color application (right), as described in the text. Lower Panel: Composite karyotype showing G-banded and pseudo-colored chromosomes. Numerical and structural chromosomal alterations are described in the text.

Figure 4. G-banded Karyotype of the 1535N Cell Line. G-banded metaphase chromosomes from 1535N cells were prepared as described in the text. M2 = der (18); M3 = add(19)(q13); M4 = der(11)t(10,11)(qter > q21..q24?q13::q22 > pter), and M6 = del(10)(q21).

Figure 5. Structural Alterations Involving Chromosome 8. Structural alterations of chromosome 8, including i(8q) chromosomes, are shown for 1542T cells (LEFT) and 1532T cells (RIGHT).

TABLE I

Allelic Status of Chromosome 8 Loci*

LOCUS	D8S504	D8S277	D8S549	D8S261	NEFL	D8S540	D8S513	D8S535	D8S505	D8S87	D8S1121	D8S255	D8S531	D8S519
cM	0.0	8.4	30.7	35.8		60.0	60.0	60.0	60.0			64.0	65.7	65.8
CHROM. LOC.	8pter	8p23	8p23	8p22	8p21	8p12				8p12			8q12	8q12
1532T	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1542T	1	1	1	1	1	1	1	1	1	1	1	1	2	2
1535T	1	1	2	2	2	2	2	2	1	2	2	2	2	2

*The allelic status of each locus is denoted by "1" (homozygous) or "2" (heterozygous).
Where known, the genetic distance (cM) and cytogenetic localization are shown.

TABLE II

Karyotypic Analysis of Immortalized Normal and Malignant Prostate Epithelial Cell Lines*

1532T
44~47,XY,i(8)(q10),+20 [2]/
46~47,idem,dup(11)(q13q23) [3]/
46~47,idem, dup(11),ins(17)(q21p11.2p13) [5]

1535T
46,XY,der(11)?qdp(q13q23)t(11;20)(q23;q11),der(20)t(11;20)(q13;q13.3)qdp(11)(q13q23) or hsr(11) [6]/
46,idem,der(3)t(3;11)(p21;q13),del(18)(q21) [4]

1542T
46,XY,der(8;20)(q10;p10),der(11)qdp(q13q23)t(11;20)(q23;q11) [4]/
46~47,idem,i(8)(q10) [2]/
36~51,idem,der(22)t(11;22)(q14;p11)t(11;20)(q23;q11.2) [2]/
45~47,idem,-der(8;20), der(8;21)(p10;q10) [2]

1535N
45-48, XY, -18, der (18) [9]/
45-48, idem, -19, add(19)(q13) [8]/
45-48, idem, -11, der(11)t(10,11)(qter > q21..q24?q13::q22 > pter)[4]/
45-48, idem, -10, del(10)(q21)[3]

*Many non-clonal chromosomal changes were also noted in all cell lines.

1532T Spectral Karyotype Composite

FIGURE 1

1542T Spectral Karyotype Composite

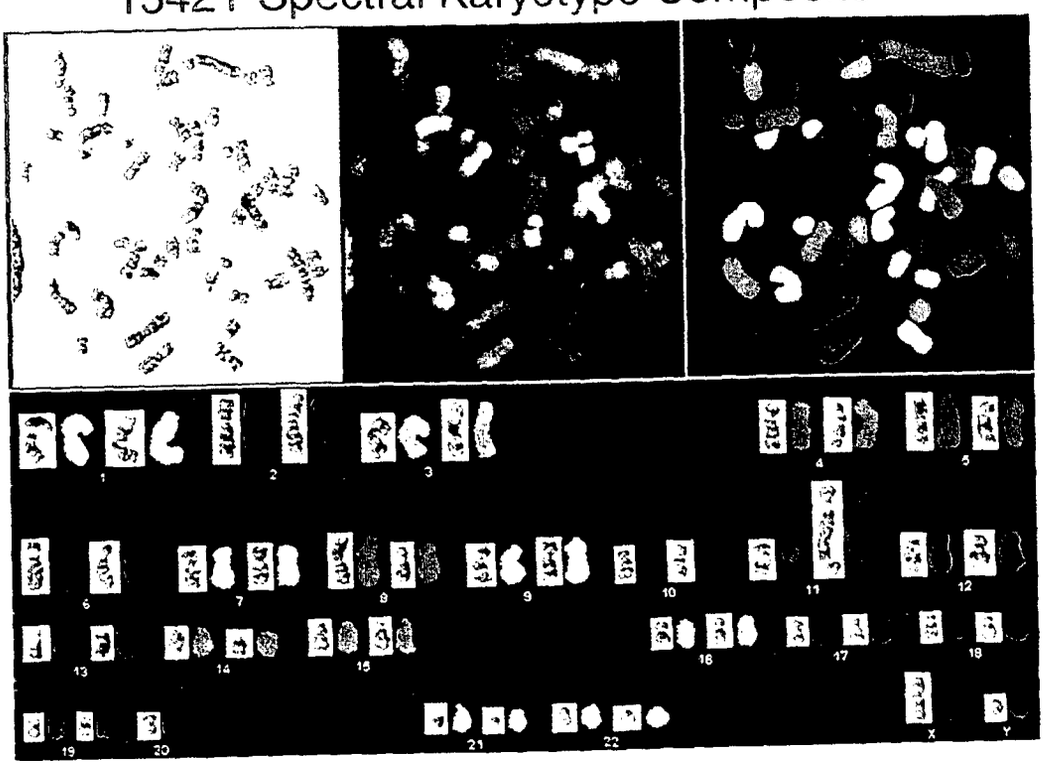

FIGURE 3

1535N

FIGURE 4

1535T Spectral Karyotype Composite

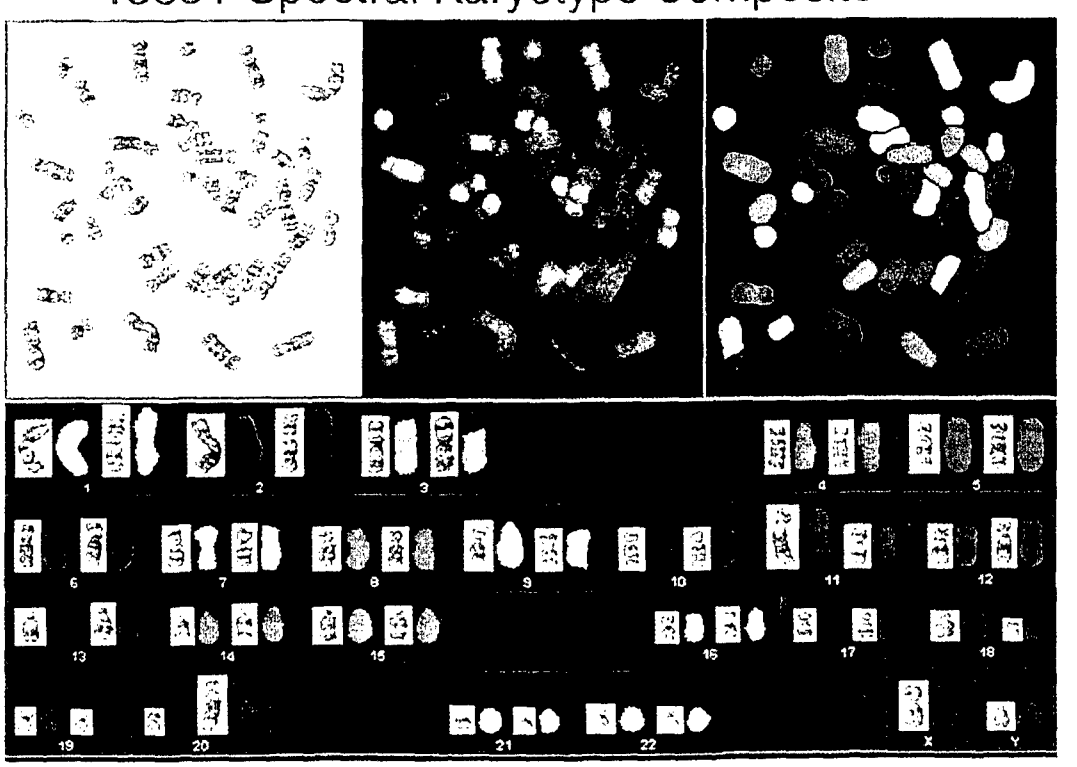

FIGURE 2

Structural Alterations Involving Chromosome 8

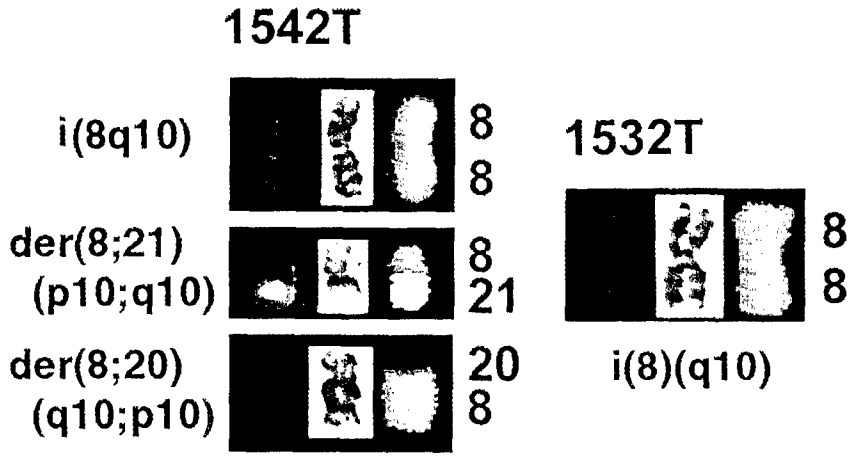

FIGURE 5

Examination of fourteen early stage prostate cancer patients by comparative genomic hybridization and interphase fluorescence *in situ* hybridization.

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Key words: molecular cytogenetics, chromosome 8, instability, heterogeneity.

Running title: CGH and FISH in early stage CaP patients.

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ABSTRACT

BACKGROUND: Although prostate cancer (CaP) continues to be the leading cancer incidence in men in North America, little is known about its etiology. Understanding the early events in CaP tumorigenesis will lead to identification of a diagnostic progression marker and decreases in treatment costs and patient morbidity and mortality.

METHODS AND RESULTS: We examined the utility of comparative genomic hybridization (CGH) on bulk-extracted genomic DNA from fourteen early stage CaP patient samples. CGH results for 12/14 (86%) patients were apparently normal, while 2/14 (14%) patients exhibited copy number gain of chromosome 8q and loss of 8p. Subsequent interphase fluorescence *in situ* hybridization (FISH) of short-term culture patient material using a combination of centromere 8 (CEP8) and *MYCC* (8q24) probes demonstrated a low-level of chromosome 8 trisomy and genomic heterogeneity. *MYCC* was always observed to correlate in a 1:1 ratio with CEP8. Interphase FISH of the three CaP cell lines, LNCaP, DU145, and PC-3, using CEP8/D13S319 (13q14) also demonstrated genomic heterogeneity within the cell lines.

CONCLUSIONS: Chromosome 8 aberrations are present at a low degree in early stage CaP tumorigenesis as evident by CGH and interphase FISH analysis. However, without adequate microdissection of the tumor material, it is difficult to assess chromosomal aberrations in early stage CaP patients by CGH unless the sample is highly enriched for homogenous tumor cells. The heterogeneity evident by interphase FISH may be due to an underlying chromosomal instability (CIN) phenotype that further precludes CGH analysis while implicating a role for the mitotic machinery in CaP tumorigenesis.

INTRODUCTION

In North America, prostate cancer (CaP) is the leading cancer incidence in men and the second most common cause of male cancer mortality [1]. While the etiology of CaP remains unknown, both environmental and genetic contributions have been associated with its carcinogenesis [1, 2]. However, our understanding of the molecular genetic changes that underlie the progression of this disease remains at an early stage, as CaP exhibits both inter- and intratumor genotypic and phenotypic heterogeneity that complicates molecular and histopathological assessment and outcome prediction [3-6]. Clinically, localised CaP is often slow-growing and latent and its diagnosis sometimes may not even impact survival for 10 to 15 years, further complicating disease assessment and prognosis [2, 7, 8]. In a substantial number of cases, however, the disease progresses to advanced stages. Advanced androgen-refractory disease is ultimately incurable and terminal. Identification of an early stage CaP-specific progression marker will allow delineation of tumor subsets that will stay indolent requiring no clinical intervention, and those that will progress to metastasis.

Progress in identifying consistent structural rearrangements in CaP has been slow. Furthermore, no consistent picture of the total chromosomal aberrations in CaP has emerged and it remains conceivable that technical limitations on the quality of the cytogenetic preparations derived from primary tumor material have precluded identification of causative chromosomal alterations in this tumor. Genetic dosage changes can be analyzed easily by comparative genomic hybridization (CGH) [9]. CGH studies of CaP that have been published generally have examined late stage, metastatic disease [10-13]. These studies have shown that there are frequent and many chromosomal aberrations in late stage CaP tumors, and that chromosomal copy number losses are five times more prevalent than gains [10].

Currently, major efforts to understand the carcinogenesis of CaP have been based on the subchromosomal level of changes, such as mutations and deletions. However, it is becoming increasingly important to examine aberrations at the chromosomal level that contribute to the

genomic instability leading to neoplasia and progression [14]. Genomic instability may be genotypically expressed as 1) microsatellite instability (MIN) as a result of failing DNA repair at the nucleotide level leading to replication errors (RER) [14, 15]; and 2) chromosomal instability (CIN) due to aberrations in the mitotic machinery leading to chromosomal copy number and structural changes, that ultimately lead to aneuploidy and destabilization of the tumor karyotype [14-16]. Thus while research effort has concentrated on examining loss of heterozygosity (LOH) at various loci and the multistep accumulation of genetic changes [6, 17-26], there has been relatively little analysis of CIN, either in CaP cell lines or fresh tumors.

In order to investigate the karyotypic changes present in early stage CaP and to determine whether CIN is a feature of CaP, we have used the genome-wide scanning technique of CGH in combination with interphase nuclei fluorescence *in situ* hybridization (FISH) in patients having low stage (pT1-T2) and grade tumors.

In this study, we have applied CGH and interphase FISH to 14 early stage CaP specimens in order to: (1) evaluate the utility of CGH for examining bulk-extracted genomic DNA from early stage CaP specimens as a prognostic indicator of tumor progression; (2) identify all regions of chromosomal gain and loss present in each patient sample; (3) determine if there are any consistent dosage changes, common to the patient cohort; (4) examine the issue of CIN in early stage prostate tumors; and (5) verify any aberrations found by CGH using interphase FISH.

MATERIALS AND METHODS

Tissue Accrual, Tissue Culture, and Cytogenetic Preparations

Prostate cancer patients who underwent radical prostatectomy at the University Health Network (Toronto, Ontario, Canada) and had no previous radio- or chemotherapy were evaluated for study eligibility based on tumor stage (pT1-T2), prostate-specific antigen (PSA) levels, and past biopsy history. The surgeon (MASJ, MB, JT) dissected a small wedge (approximately 1-2 cm^3) of tumor tissue from the excised prostate, and the resected prostatic capsule was analyzed for extracapsular tumor extension by the pathologist (JMS). The tissue wedge was quick-sectioned and hematoxylin and eosin (H&E)-stained. Samples assess by the pathologist as having high tumor volume (> 80%) within the surrounding normal stroma were designated for study inclusion. Subsequently, a mirror-image tissue wedge from the directly adjacent face was dissected from the prostate and returned to the laboratory for research. This sample was divided to several equal-sized portions. Two tissue pieces were snap-frozen in liquid nitrogen for future archival use. Another piece was placed in DNA extraction buffer for CGH analysis. Extraction of genomic DNA from tumor tissue specimens followed standard protocols [27, 28], and routinely yielded approximately 200ug of high molecular weight DNA. The remainder of the tissue was subdivided for cytogenetic preparations.

Short-term cultures (< 1 week) were prepared by dissociating the tissue pieces into fine single cell suspensions using a cross-blade disaggregation method plus 250U/ml collagenase IV (Gibco, BRL) in tissue culture media (RPMI 1640, 10% fetal bovine serum, antibiotics) for 2-3 hours. This cell suspension was centrifuged gently and washed with phosphate buffer solution, seeded into tissue culture flasks for overnight attachment (usually 1-3 days) and subsequently harvested for interphase FISH analysis (described below). Harvested cytogenetic preparations were not obtainable from Patients 2 and 7, as they did not grow in culture. There was not enough material from Patient 14 for tissue culture.

Comparative Genomic Hybridization (CGH)

CGH was performed as previously described [9, 29]. Briefly, 2ug each of normal and tumour DNAs were labelled by nick translation with Digoxigenin-11 dUTP (Boehringer Mannheim) and Biotin-14dATP (Gibco/BRL) respectively. Final labeled DNA fragments ranged between 500 bp to 2kb. Equal amounts of labeled tumor and normal were co-precipitated in the presence of excess 10ug Human Cot-1 DNA (Gibco/BRL) and resuspended in a hybridization buffer (Hybrisol VII, Oncor, Gaithersburg MA), denatured and hybridized to denatured normal male metaphase slide preparations for 72 hours at 37°C. Post hybridization washed and detections were carried out as previously described with the tumour genome detected with green-fluorescing FITC and the normal genome detected with red-fluorescing rhodamine. Computer analysis of the resultant green:red fluorescence intensity ratio along the length of each chromosome reveals the copy number changes specific to each locus [29-31]. The green and red fluorescence intensities at each locus are directly dependent upon the test and reference DNA abundance at each locus. The normal range for the green:red ratio is between 0.8 – 1.2 [30, 31]. At any locus, a green:red ratio greater than 1.2 is indicative of copy number gain of the tumor DNA. Similarly, a green:red ratio less than 0.8 indicates copy number loss of tumor DNA. A ratio greater than 1.5 is indicative of high level amplification. Because frequently genomic DNA is obtained from a heterogeneous population of tumor cells, CGH emphasizes the homogenous aberrations in the population while averaging any heterogeneous changes [9, 29]. Unfortunately, the resolution for amplification and deletion detection by CGH is quite low; this corresponds to a region of approximately 2 - 15Mb (copy number multiplied by amplicon size) and 10 - 20Mb [9, 32] in size, respectively. Images were captured using the Vysis PathVysion software, and analysis performed using the Vysis Karyotype software (Vysis, Inc. Downers Grove, MI). Results at telomeric or centromeric regions due to the presence of highly repetitive genomic sequences at these sites were not analysed. Ten metaphases were analysed to create the final CGH profile with 99% confidence intervals. Negative and positive controls for CGH analysis were performed using normal male genomic DNA, and the

IMR32 neuroblastoma cell line, respectively. IMR32 has been previously characterised in our laboratory.

Fluorescence in situ *Hybridization (FISH)*

Harvested cytogenetic preparations from patients 1 – 13 (CaP1 – CaP13), excluding CaP2 and CaP7, were dropped onto glass slides as per standard protocols and previously described [33] using 1.5 hour colcemid treatment and 75 mM KCl hypotonic treatment. Normal cytogenetic control slides were made from phytohaemaglutinin-stimulated normal male lymphoblasts and harvested as above. Denaturation of the centromere enumeration probe 8 (CEP8) and 8q24 (*MYCC*) FISH probes (Vysis Inc., Downers Grove, MI) and application of the probe(s) to the slides were as per manufacturer's instructions (Vysis Inc. Downers Grove, MI). At least 110 nuclei were used for enumerating the co-hybridized probes for each sample. Images were viewed and captured using the Vysis PathVysion softwre and stored on a Power Macintosh workstation. Follow-up FISH analysis was also carried out using the CEP8/D13S319 (Vysis Inc.) probes.

CaP Cell Lines

LNCaP (CRL-1740), DU145 (HTB-81), and PC-3 (CRL-1435) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). LNCaP, an androgen-dependent cell line originating from a lymph node metastasis [34, 35], was grown in RPMI 1640 with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. DU145, an androgen-independent cell line obtained from a metastasis to the bone [36], was grown in F15K Minimum essential medium with 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. PC-3, also an androgen-independent cell line and originated from a brain metastasis [37], was grown in Ham's F12K with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum. Cell lines were harvested as described above, and used for dual-color FISH analysis using the combination CEP8/D13S319 probes.

RESULTS

Comparative genomic hybridization analysis of the fresh tumor samples revealed normal karyotypes in 12/14 (CaP1 - CaP12) samples examined. CaP14 revealed gain of the long arm of chromosome 8 (8q), and loss of the short arm of chromosome 8 (8p), suggestive of isochromosome 8q formation. CaP13 revealed gain of 8q and loss of 8p. The positive control IMR32 neuroblastoma line prepared for CGH showed high-level amplification at the 2p22 and 2p24 chromosomal regions, as expected. The negative control (normal male DNA) showed normal CGH results. Three representative CGH profiles from patients 10, 13, and 14 are shown in Figure 1.

Dual-color interphase FISH was used to examine the patient material and cell lines on a cell-by-cell basis in order to investigate the chromosome 8 aberrations identified by CGH in two of the patient samples (Figure 2). Centromere 8 probe (CEP8, green) was used together with *MYCC* (8q24, orange) probe to interrogate the extent of chromosome 8 long arm gain in the patient samples (Table 1). *MYCC* was found to always correlate in a 1:1 ratio with CEP8 in the normal control and the patient material nuclei. Control normal male lymphoblast nuclei with CEP8/*MYCC* established trisomy 8 levels at < 1%. Trisomy of chromosome 8 was observed in nuclei derived from the cytogenetic preparations of the patient samples at a greater level than the established baseline. Heterogeneity, possibly as a result of CIN, was detected as low-level of cells monosomic and polysomic for CEP8/*MYCC*.

Interphase and metaphase FISH using CEP8 alone on LNCaP cells demonstrated the majority of cells to have polysomy 8 (for LNCaP, this was specifically tetrasomy) and a smaller population of trisomy 8 cells (Table 2). DU145 exhibited three populations of cells, having disomy, trisomy, and monosomy of CEP8. The majority of cells of PC-3 exhibited disomy and a small minority exhibited polysomy of CEP8, although there were also trisomy and monosomy cells. Table 3 lists the results of the dual-color hybridization of CEP8/D13S319 (13q14) for the

cell lines. The degree of genotypic heterogeneity for both these probes within the cell lines likely due to CIN is evident in Table 3. FISH images from these three cell lines are shown in Figure 3.

DISCUSSION

Currently, many studies suggest that CaP has extensive inter- and intratumor heterogeneity. As shown by Kallioniemi et al. [29], dilution of homogenous genomic changes in tumor cells, by CIN, tumor heterogeneity or normal stromal cell contamination, can reduce the sensitivity of CGH in discerning copy number changes. In this study, CGH showed normal karyotypes for CaP1 - CaP12. To determine whether CIN was a possible source of variation, interphase FISH analysis was performed. Interphase FISH analysis of CaP cytogenetic material (CaP1 – CaP13) revealed cell-by-cell heterogeneity at the CEP8/*MYCC* loci. Interestingly, one patient (CaP10) had a greater degree of trisomy 8 (44%), even though the CGH findings did not detect gain suggesting that CGH may not be sensitive enough to detect early chromosomal changes in CaP.

Interphase and metaphase FISH using CEP8 and CEP8/D13S319 on the cell lines (LNCaP, DU145, and PC-3) showed close agreement with recently published SKY karyotypes done by our group [38]. As expected, the majority of LNCaP cells exhibited tetrasomy 8 (Tables 2 and 3) corresponding to the established tetraploid chromosome number. Previous studies from this laboratory did not identify minor populations of trisomy 8 cells and both trisomy and monosomy of chromosome 13 that were not detectable in metaphase analysis by SKY [38] or CGH karyotypes [39]. This indicates that CIN may be an intrinsic property of this cell line. Both DU145 and PC-3 generally showed hypotriploid populations of cells using CEP8 and CEP8/D13S319. DU145 and PC-3 were assigned composite karyotypes and this degree of heterogeneity is reflected in the interphase FISH results and may further explain the variability of these cell lines observed by others [36, 37].

Allelotyping experiments have demonstrated frequent involvement of chromosome 8 in CaP tumorigenesis, and analysis of extensive loss of heterozygosity (LOH) loci along 8p in CaP patients [6, 17, 18, 26] have narrowed three tumor suppressor gene loci along the region 8p12, 8p21, and 8p22. Furthermore, recent studies by Macoska et al. [40] and Virgin et al. [25] using human papillomavirus (HPV) E6/E7 and simian virus 40 (SV40) Large T antigen immortalised CaP

patient cell lines, showed a direct correlation between 8p LOH allelotyping data and isochromosome 8q formation or other structural rearrangements of 8p by molecular cytogenetics. However, it was also shown that 8q gain could occur independently of 8p loss through complex structural rearrangements [40]. Alers et al. [41] demonstrated by FISH in localised prostate tumors, lymph node metastases, and distant metastases samples that +8 was more frequent than –8. Subsequent examination of the lymph node metastasis sample by both CGH allowed correlation of +8 by interphase FISH with 8q gain as determined by CGH, and conversely –8 by FISH with 8p loss by CGH [41]. Together, the data suggest that 8q gain may be independent of and contributes to the 8p- genotype in the tumorigenic process, but can also sometimes occur through isochromosome 8q formation. Interestingly, by CGH two patients (CaP13, CaP14) from our study cohort exhibited chromosome 8q gain and 8p loss. Interphase FISH results using the probe combination CEP8/*MYCC* suggest that the aberration mechanism not only occurs via isochromosome 8q formation, but can also proceed as a two step process involving 8q gain and 8p loss.

In conclusion, it is apparent that without microdissection of tumor cells from the normal surrounding stromal bed, CGH from bulk dissected fresh tissue may be unsuitable as a prognostic tool for detecting chromosomal numerical aberrations. Furthermore, considerable genomic heterogeneity in both inter- and intratumor populations may significantly decrease CGH signals, often to below detectable significance levels. This may be due to an underlying CIN phenotype [15], that may play a significant role in early stage CaP tumorigenesis and may be evident using interphase FISH.

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FIGURE LEGENDS

Figure 1 – CGH profiles of CaP10; CaP13; and CaP14.

H&E staining of fresh CaP tumor (top left); CGH RGB metaphase spread (top right); CGH profile (bottom); of patients 10 (A), 13 (B), & 14 (C). While all three patient tumors exhibited high tumor volume by H&E, CGH revealed varying extents of aberration. Note the indication of 8q gain by CGH in patients 13 and 14, a finding confirmed in all three patients by FISH using CEN8 and *MYCC* (8q24) probes. Chromosomal areas of gain are over-represented by tumor DNA and have a higher green:red ratio; conversely, regions of loss are under-represented by tumor DNA and have a lower green:red ratio. Because frequently genomic DNA is obtained from a heterogeneous population of tumor cells, CGH emphasizes the homogenous aberrations in the population while averaging any heterogeneous changes [9]. This averaging includes the signal-reducing effects of sample contamination by normal cells.

Figure 2 – Interphase FISH images from fresh tumor samples A) CaP10; B) CaP13.
Dual-color FISH images using CEP8 (red) and *MYCC* (8q24; green) demonstrate representative enumerated nuclei. Note that *MYCC* hybridization always correlated with CEP8 in a 1:1 ratio.

Figure 3 – Interphase and metaphase FISH images from the prostate cell lines LNCaP (A); DU145 (B); PC-3 (C).
Dual-color interphase FISH using CEP8 (red)/D13S319 (13q14; green) in the cell lines revealed cell-by-cell heterogeneity.

TABLES

Sample	n	Normal 8/*MYCC*	Trisomy 8/*MYCC*	Monosomy 8/*MYCC*	Polysomy 8/*MYCC*
Normal Control	179	96%	< 1%	3%	< 1%
Patient 1	179	88%	8%	1%	3%
Patient 3	201	83%	5%	2%	1%
Patient 4	113	85%	9%	6%	0
Patient 5	133	89%	5%	2%	2%
Patient 6	128	90%	4%	5%	< 1%
Patient 8	147	90%	8%	< 1%	2%
Patient 9	118	92%	6%	4%	< 1%
Patient 10	111	48%	44%	4%	4%
Patient 11	124	90%	6%	4%	1%
Patient 12	148	90%	5%	5%	0
Patient 13	121	70%	14%	13%	2%

Table 1 – FISH CEP8/*MYCC* results for CaP patients. Note *MYCC* always correlated in a 1:1 ratio with the CEP8 hybridization pattern.

Sample	n	Normal 8	Trisomy 8	Monosomy 8	Polysomy 8
Normal Control	179	96%	< 1%	3%	< 1%
LNCaP	167	1%	20%	< 1%	78%
DU145	110	47%	31%	21%	< 1%
PC-3	192	77%	6%	4%	13%

Table 2 - FISH CEP8 results for the three metastatic CaP cell lines.

	8 Centromere/D13S319 (13q14) Copy Number								
Cell Line	2/2	2/3	3/1	3/2	3/3	4/2	4/3	4/4	Others
Normal Control	97%	-	-	-	-	-	-	-	3%
LNCaP	-	-	-	6%	7%	5%	66%	5%	10%
DU145	-	-	-	63%	4%	17%	3%	< 1%	11%
PC-3	43%	29%	-	1%	4%	-	-	5%	18%

Table 3 - FISH CEP8/D13S319 (13q14) results for CaP cell lines.

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Figure 1

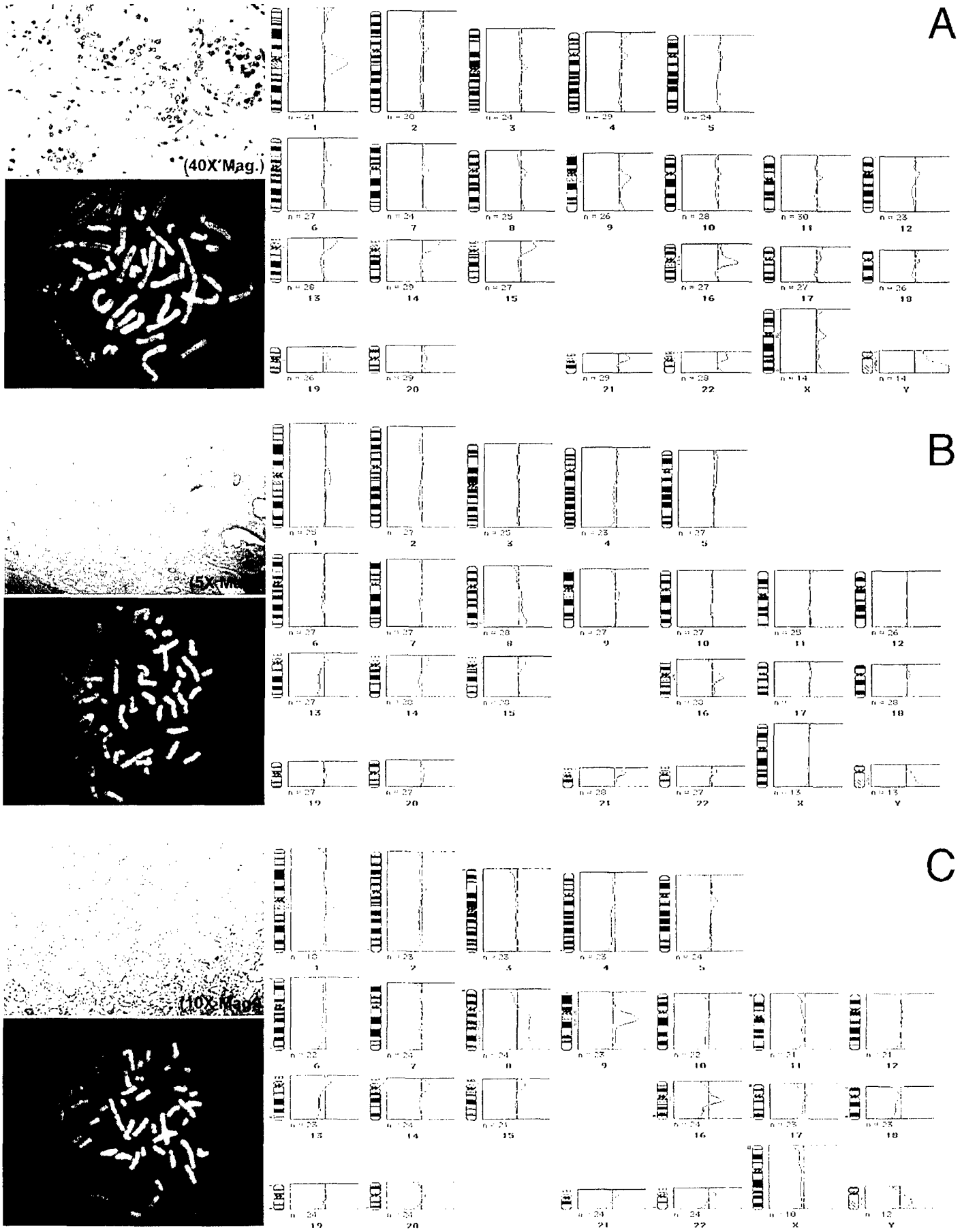


Figure 2

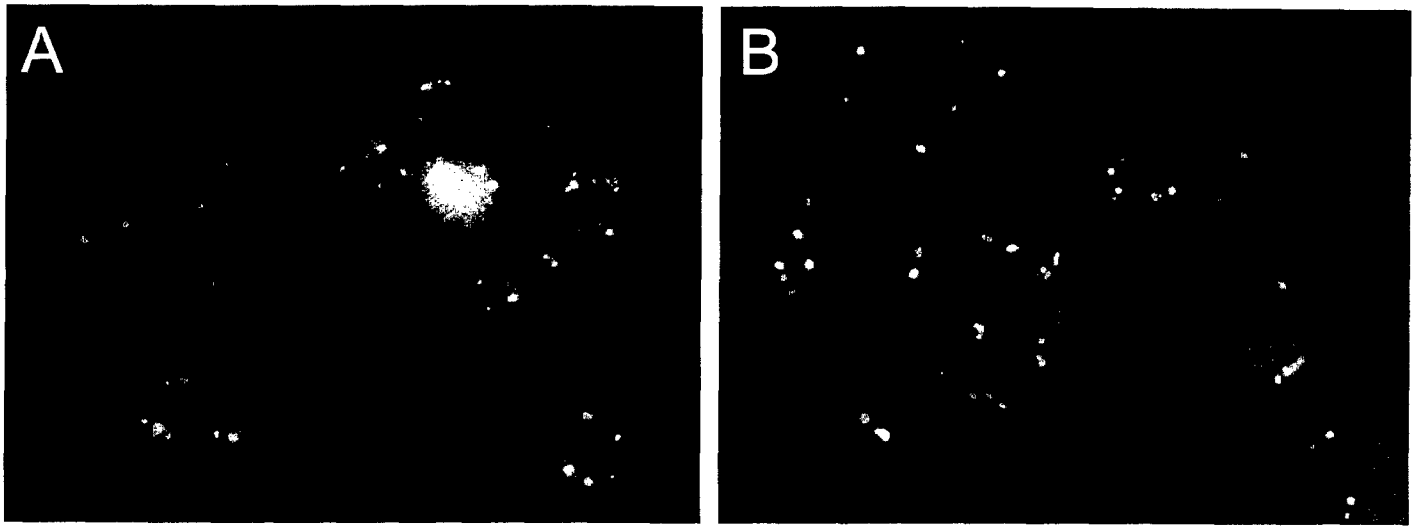
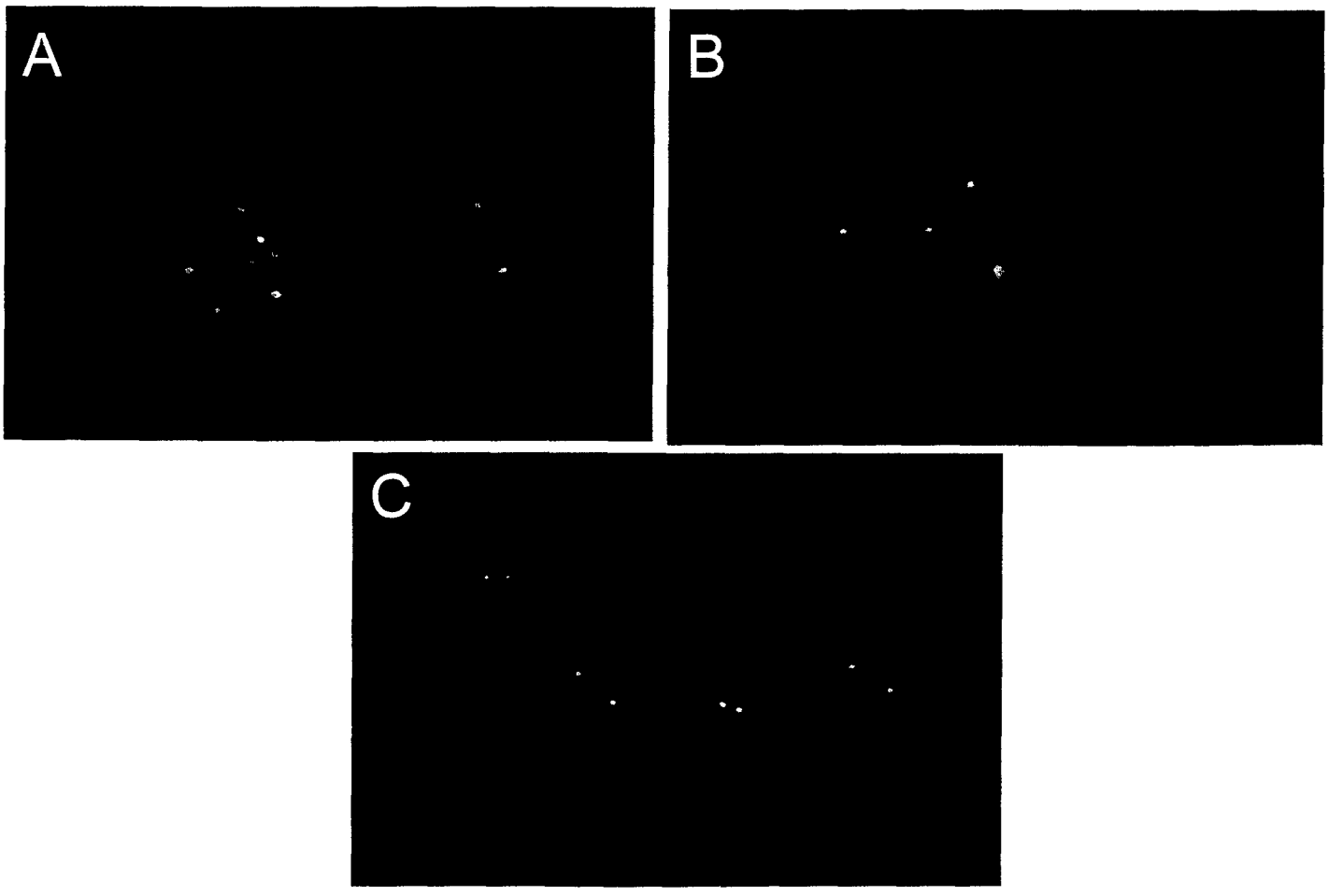


Figure 3



Appendix 4

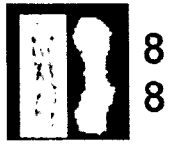
Table Summary of karyotype and SKY findings on direct harvests of prostate tumors

Case No.	Stage	G-banding result	SKY result
CaP J-3	T2	1x47,XY,-19,+M,+M/1x46,XY	
CaP J-7	T2a	1x46,XY,der(1),-9,der(15),?der(17)/19x46,XY	
CaP J-10	T2a		1x46,X,-Y,+7/9x46,XY
CaP J-13	T2	1x46,XY,add(13)(p10),?der(19)/19x46,XY	
CaP J-15	T2		2x92,XXYY
CaP J-20	T2	1x45,X,-Y,der(12)t(3;12)/1x47,X,-Y,+3,+15/ 8x45,X,-Y	

Appendix 4 - Summary of Chromosome 8 Findings Using Different Molecular Cytogenetic Methods

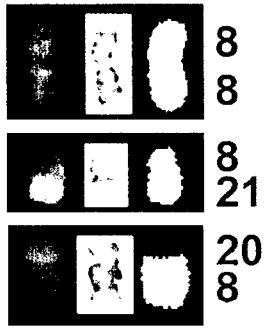
A

1532T P44



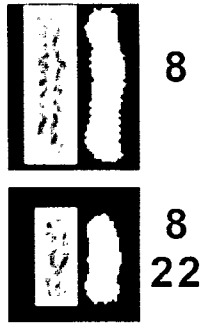
B

1542T P44



C

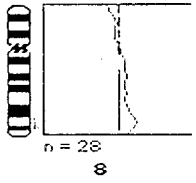
PrEC-T P27



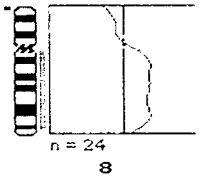
D

Fresh Tumor

Patient 13



Patient 14

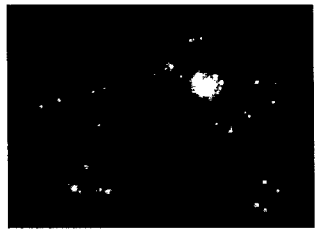


E

Fresh Tumor

Patient 13

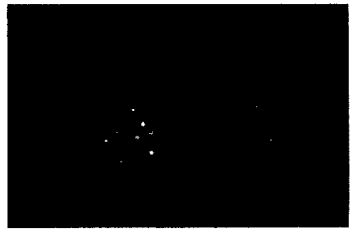
Patient 10



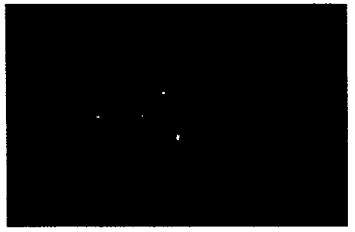
F

CaP Cell Lines

LNCaP

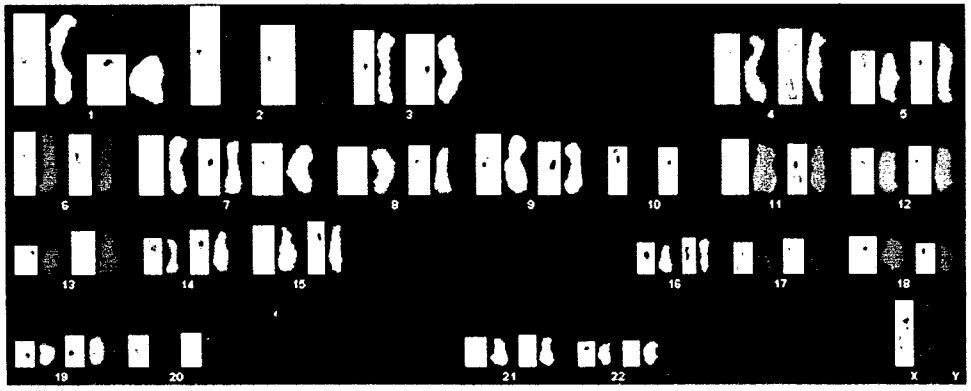


DU145



PC-3

G



Appendix 5

Genomic alteration in prostate intraepithelial neoplasia can be predictive of more aggressive disease process

(Manuscript in preparation)

To test our hypothesis that genomic alteration in prostate intraepithelial neoplasia (PIN) cauld be predictive of more aggressive disease process, we applied interphase FISH on prostate biopsies of archival paraffin embedded material from patients with initial diagnosis of high grade PIN (HPIN) comparing two groups of patients as follows:

A) Group A: patients with HPIN as a primary diagnosis in prostatic biopsies and did show invasive carcinoma on subsequent biopsies.

B) Group B: patients with HPIN as a primary diagnosis in prostatic biopsies and did not show evidence of carcinoma on subsequent follow-up biopsies.

These two groups have been matched for age, PSA level and for rectal examination findings.

All the H&E slides of the cases diagnosed as HPIN between 1995-1997 in the record of The University Health Network have been reviewed by 2 pathologists to confirm the diagnosis and to determine the adequacy of the specimen for FISH analysis. Only those with sufficient material were included in the study. Interphase FISH have been performed on 5-micron unstained tissue sections using adjacent H&E stained sections as guidance.

The standard technique for FISH on paraffin sections was applied [1, 2,3,4, 5] with some modifications. Tissue sections have been deparaffinized in xyelen for 10

minutes, dehydrated in 100% ethanol for 10 minutes, incubated in 2x SSC for few minutes, digested in pepsin solution (pH 1.5) for 12-16 minutes in at 45° C, rinsed in 2x SSC at room temperature for 5 minutes, and air-dried. Biotin labeled cosmid probe for chromosomes 8 and directly labeled VYSIS CEP probes for chromosomes 7, 10, and 4 have been used. Dual-probe hybridization has been performed in some cases. One microliter of the probe was mixed with 7 µl spectrum CEP hybridization buffer and 2 µl of deionized water. The probe mixture was applied to the slides and sealed by rabber cement. Co-denaturation was performed using the HYBrite at 88° C for 8 minutes followed by hybridization at 37° C for 30 hours. Unbound probe was removed by washing the slides at in 0.4 x SSC, 0.3% NP-40 at 72 °C for 2 minutes and then in 2x SSC, 0.1 NP-40 at room temperature and air-dried. The nuclei were counterstained with DAPI.

Scoring criteria: For each probe the number of FISH signals in 100 nonoverlapped intact (spherical) interphase nuclei from foci of HPIN has been counted by two independent investigators. The number of signals per nucleus has been scored as (1, 2, 3, 4, and >4 signal per nucleus. Nuclei from stromal element have not been enumerated. FISH by using a centomere probe for chromosome 4 was used as a negative control.

Criteria for evaluation of numerical chromosomal anomalies: Due to truncation of artifactual loss of signals is expected; however we have applied a very conservative criteria to detect any significant true numeric changes. Our criteria to evaluate numeric chromosomal abnormality is as follows:

1) Chromosomal gains have been diagnosed when more than 8% of the nuclei exhibit more than two signals.

2) Chromosomal losses have been diagnosed when more than 50% of the nuclei

exhibit a reduction of signal number.

3) Tetraploidy has been assumed when all chromosomes investigated show signal gains up to four. These cutoff values were adopted from the available literature [6-9].

Result: We have successfully performed interphase FISH analysis on slides from 28 patients (12 from group A and 16 from group B) for chromosomes 4, 7, 10 and chromosome 8 for 12 patients (4 patients from Group A and 8 patients from Group B) Our preliminary findings indicated the presence of different chromosomal anomalies in 4/12 (33%) cases of the group A and in 2/16(12.5%) cases of group B. All the chromosomal changes were detected in a form of a gain and no chromosomal losses have been identified in any case. Gain of chromosome 7 has been seen in 3/28 case, gain of chromosomal 10 in 2/26 cases and gain of chromosomal 8 in 2/12 case. No numeric chromosomal changes have been seen in chromosome 4. No numeric chromosomal anomalies have been noticed in the adjacent hyperplastic or normal prostate glandular epithelium. By applying the same cytogentic technique on 5-micron paraffin-embedded sections from TURP specimens from patients with nodular hyperplasia (BPH) using directly labeled VYSIS CEP probe for chromosome 8, there were no chromosomal numeric anomalies in any of the 12 specimens that have been examined.

Conclusion: These preliminary results indicate that although no single numerical chromosomal anomaly could be assigned as a predictor for the progression of HPIN to carcinoma in the subsequent follow-up biopsies, however it is evidentely that the overall numeric chromosomal abnormalities are more common in HPIN from patients who showed carcinoma in the subsequent follow-up biopsies. This suggests that chromosomal instability is more common in HPIN foci which progress to or adjacent to prostate carcinoma.

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Appendix 5

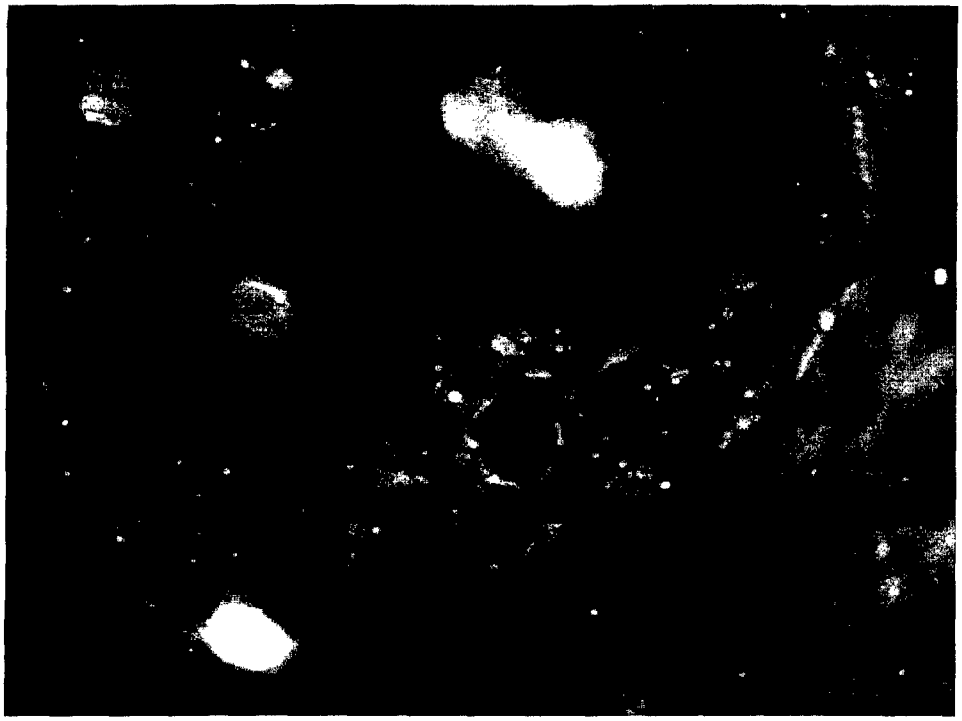


Figure. Two color FISH analysis of paraffin section of HPIN lesions. Note overrepresentaion of chromosome 8 signals (green) in comparison to chromsome 4 (red).

Appendix 6 - Development of Microarray Analysis of PCa

Figure 1 - LCM-DOP PCR

Prostate tumors typically present as numerous microfoci of epithelial cells supported by the stroma. In studying the genomic makeup of PCa, genomic heterogeneity between individual foci, and contamination by normal epithelial cells are inherent confounding issues. To circumvent this problem, we are employing laser capture microdissection (LCM) to obtain a pure, homogenous population of epithelial cells. This technique uses laser pulses to adhere select cells of interest in a H&E stained tissue section, onto a plastic film (A). Panel B shows an H&E section of prostate tumor before and after microdissection. The isolated cells are digested with protease to liberate the DNA, which can be subsequently amplified by PCR using degenerate oligo primers (DOP-PCR). Panel C shows the product of DOP-PCR, using genomic DNA isolated from peripheral blood. Lane 1, 1 kb ladder; lane 2, undigested genomic DNA template; lanes 3-6, products of DOP-PCR after 2 rounds of amplification (see attached protocol). Work is in progress to optimize the amplification conditions for microdissected samples, and to generate probes suitable for use in CGH analysis.

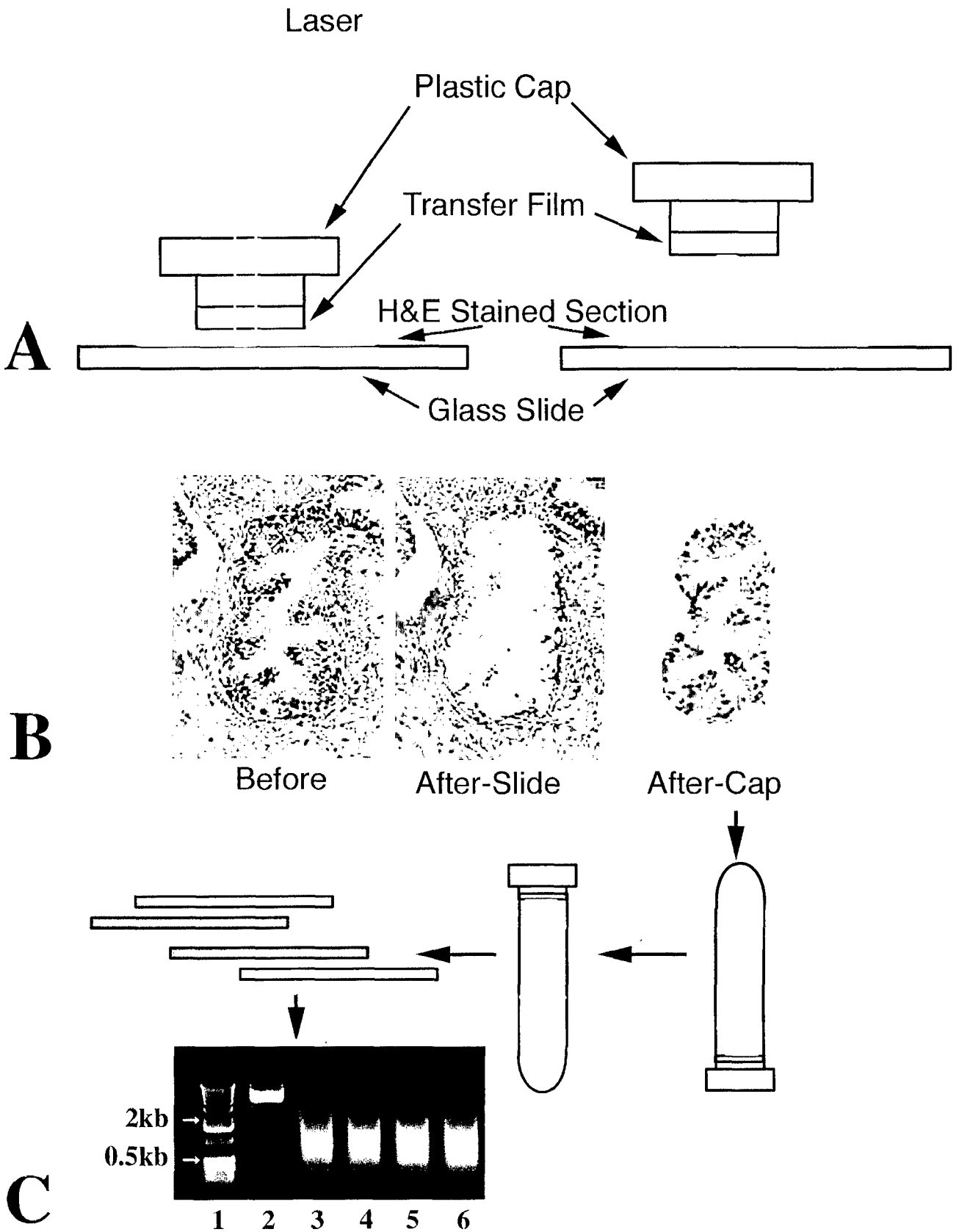
Figure 2 - Glass Microarrays

In addition to genomic alterations, changes in the expressions of a set of genes may also exhibit association with the progression of CaP. Such changes, if present in early stages, may herald the commitment of the cells into an aggressive phenotype and thus be of prognostic value. In a parallel study (funded by institutional support), our laboratory has been investigating the differential gene expression between prostate cell lines with varying phenotypes. For this, total RNA extracted from PC-3 and DU145 cell lines were labeled with Cy3 (red) and Cy5 (green) conjugated dCTP, respectively, and used to screen an "in house" gene chip of 1700 ESTs (Panel A). Comparison of the expression levels, as relative fluorescence intensities identified 93 ESTs to be overexpressed in PC-3 cells and 942 to be overexpressed in DU145 (Panel B). Work is in progress to further compare the expression patterns in these cell lines with that of the less aggressive, hormone-dependent cell type, LNCaP. We also plan to extend this study to compare tumor samples derived from a patient cohort of two age and stage matched populations, one which exhibits disease recurrence during 5 years of follow up post radical prostatectomy, and the other which remains disease free. Future work will also employ an expanded array of 20000 ESTs.

Table 1 - Results from Microarray Analysis

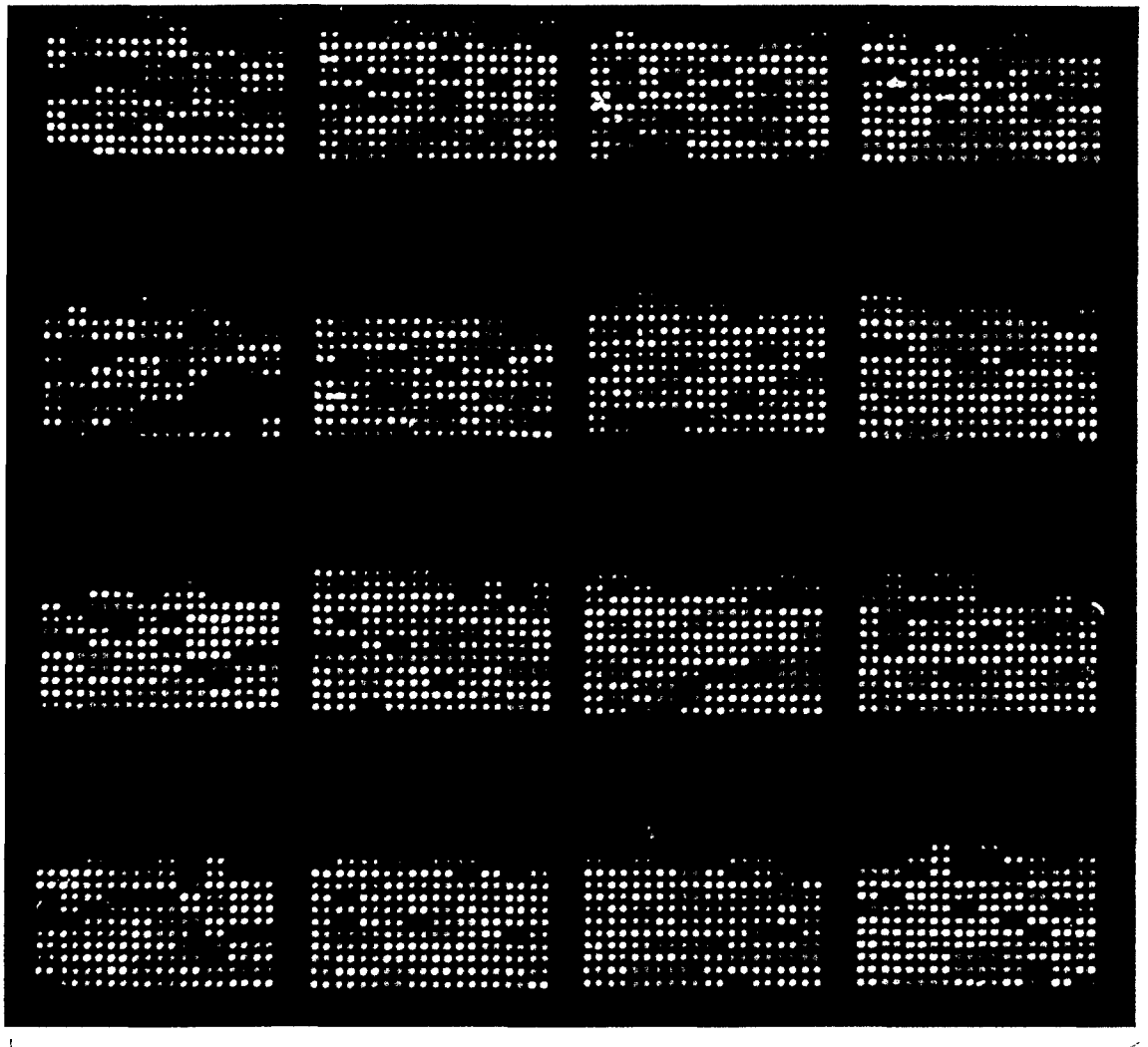
This table reports the ten most highly expressed and the least expressed genes from the scatterplot analysis of the DU145 vs PC-3 microarray (Figure 2B).

APPENDIX 6 - Figure 1

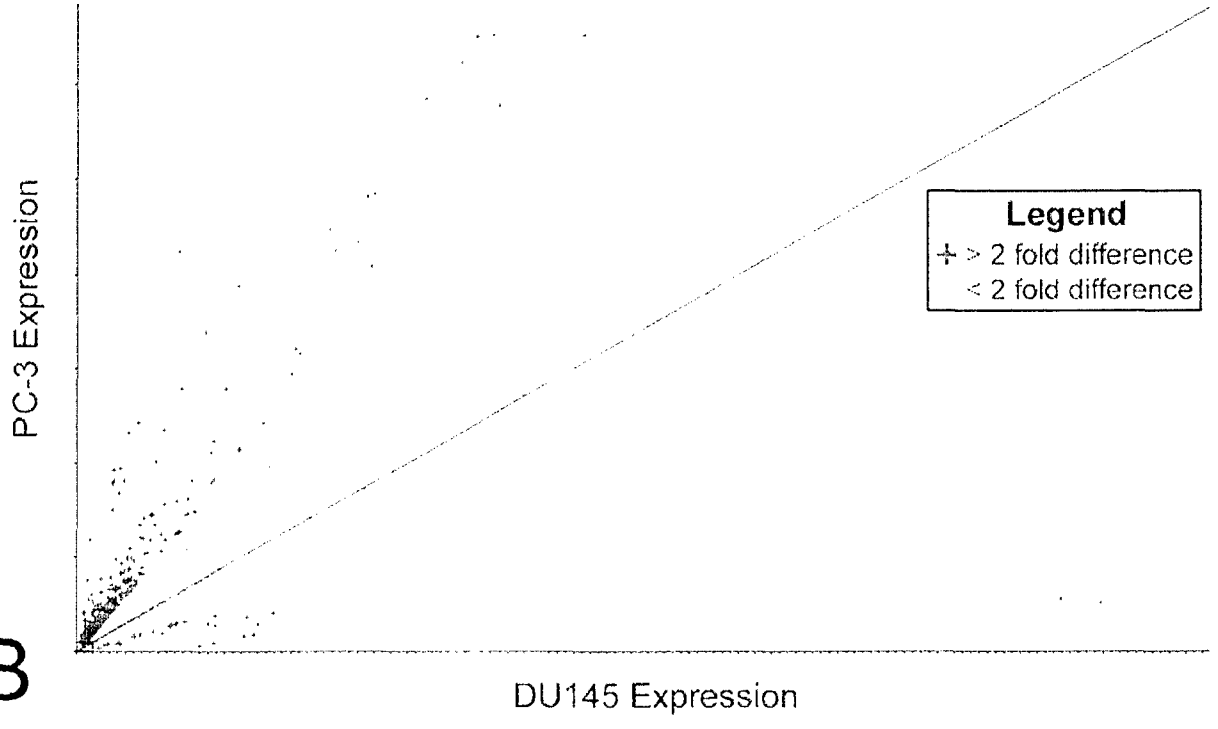


APPENDIX 6 - Figure 2

A



B



Appendix 6 - Table 1

	Array Row	Array Column	Row	Column	Name	Ratio DU145/ PC-3
DU145 > PC-3	1	4	3	10	H04204::SIGNAL TRANSDUCER CD24 PRECURSOR.	13.23
	2	4	5	4	N42770::TYROSINASE PRECURSOR (EC 1.14.18.1) (MONOPHENOL MONOOXYGENASE).	13.24
	2	1	9	10	AA088648::BASIC FIBROBLAST GROWTH FACTOR RECEPTOR 1 PRECURSOR (BFGF-R) (EC 2.7.1.112) (FMS-LIKE TYROSINE KINASE-2) (C-FGR).	13.79
	4	3	1	14	T65624::DUAL SPECIFICITY PROTEIN PHOSPHATASE 6 (EC 3.1.3.48) (EC 3.1.3.16) (DUAL SPECIFICITY PROTEIN PHOSPHATASE PYST1).	18.42
	3	3	3	20	R70894::5'-TG-3' INTERACTING FACTOR (HOMEOBOX PROTEIN TGIF).	18.63
	2	4	1	11	R34413::POLY (ADP-RIBOSE) POLYMERASE (EC 2.4.2.30) (PARP) (ADPRT) (NAD(+) ADP- RIBOSYLTRANSFERASE) (POLY(ADP-RIBOSE) SYNTHETASE).	18.86
	1	4	1	6	T78285::DEATH DOMAIN CONTAINING PROTEIN CRADD (CASPASE AND RIP ADAPTATOR WITH DEATH DOMAIN) (RIP ASSOCIATED PROTEIN WITH A DEATH DOMAIN).	19.60
	4	3	1	15	T78208::RAS GTPASE-ACTIVATING-LIKE PROTEIN IQGAP1 (P195) (KIAA0051).	23.40
	4	3	3	13	T98705::1,4-ALPHA-GLUCAN BRANCHING ENZYME (EC 2.4.1.18) (GLYCOGEN BRANCHING ENZYME) (BRANCHER ENZYME).	28.92
	4	4	7	14	W00681::(Dup1)SOX-22 PROTEIN.	32.77
PC-3 > DU145	2	3	10	7	W58008::ALDOSE REDUCTASE (EC 1.1.1.21) (AR) (ALDEHYDE REDUCTASE).	0.11
	2	2	4	10	H40164::BRAIN SPECIFIC POLYPEPTIDE PEP-19 (BRAIN SPECIFIC ANTIGEN PCP-4).	0.17
	4	3	10	19	AA128375::CELLULAR NUCLEIC ACID BINDING PROTEIN (CNBP).	0.18
	4	3	8	11	W49770::HYPOTHETICAL PROTEIN KIAA0026.	0.18
	3	2	5	17	H93255::METALLOTHIONEIN-IE (MT-1E).	0.21
	4	2	6	8	W03376::NICOTINAMIDE N-METHYLTRANSFERASE (EC 2.1.1.1).	0.22
	3	2	11	13	W69515::MAP KINASE-ACTIVATED PROTEIN KINASE 2 (EC 2.7.1.-) (MAPK-ACTIVATED PROTEIN KINASE 2) (MAPKAP KINASE 2) (MAPKAPK-2).	0.22
	2	4	4	18	R81846::FERRITIN LIGHT CHAIN.	0.24
	2	4	4	15	R89564::SODIUM/POTASSIUM-TRANSPORTING ATPASE BETA-1 CHAIN (EC 3.6.1.37) (SODIUM/POTASSIUM-DEPENDENT ATPASE).	0.27
	4	3	8	2	W47644::NOVEL ANTIGEN 2 (NAG-2).	0.30

Abstract List

1. B. Beheshti, P.C. Park, L.R. Kapusta , L. Klotz, J.A. Squire. Analysis of prostate cancer cell lines and tumour using high density EST microarray. Abstract accepted in the Human Genome Meeting (HGM2000), Vancouver, BC, CANADA. April 9-12, 2000.

   Although prostate cancer (CaP) remains the number one cancer incidence in men in North America, etiology of this disease is poorly understood. Identification of a prognostic progression marker(s) in CaP is critical for treating patients at an early stage while the disease is still curable. Microarray technology allows for the simultaneous analysis of expression patterns for greater than thousands of genes in a single experiment. By examining CaP cell lines and fresh patient material using the Ontario Cancer Institute (OCI) microarrays, we have assessed differential genes expression that may be associated with the tumorigenic process. Furthermore, the importance of chromosome 8 in CaP initiation and progression is well-established. By selecting those informative genes and ESTs from microarray studies, as well as those from "hotspot" chromosome 8 loci, and others identified through datamining of biological databases, we are in the process of constructing a custom microarrays that, with refinement, will be highly prognostic for CaP progression. PCP was supported by the AUA/AFUD. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

2. B. Beheshti, J. Karaskova, B. Beatty, P.C. Park, M. Beheshti, J. Trachtenberg, J. Sweet, M. Jewett, J. Rhim, B. Hukku, J. A. Squire, J. Macoska. Chromosomal Aberrations in Early Stage Prostate Cancer Patients and Virus-Immortalised Cell Lines Identified by Combined CGH, SKY, and Allelotyping Techniques. Abstract accepted in the American Association for Cancer Research 91st Annual Meeting, San Francisco, CA, USA. April 2000.

   We have utilised comparative genomic hybridization (CGH) with confirmatory interphase nuclei fluorescence *in situ* hybridization (FISH) to examine fourteen early stage prostate cancer (CaP) patients for recurrent chromosomal copy number changes suggestive of tumor initiation and progression. In

addition, combined conventional and spectral karyotyping (SKY) techniques and allelotype analysis were used to assess numerical and structural chromosomal alterations in two cell lines derived from normal human prostatic epithelium, and three cell lines derived from primary human prostate tumor epithelium, immortalized with the E6 and E7 transforming genes of human papillomavirus (HPV) 16 or the Large T antigen gene of simian virus 40 (SV40). CGH and interphase FISH results suggested isochromosome 8q formation in the patient cohort. Interestingly, allelotyping identified loss of 8p sequences in two of the three primary prostate tumor-derived cell lines, and SKY analysis revealed that the loss of 8p sequences was directly due to isochromosome 8q formation and/or other structural alterations of chromosome 8. This provides evidence that 8p loss in primary human prostate tumors may, in some cases, result from complex structural rearrangements involving chromosome 8. Moreover, the data reported here provides direct evidence that such complex structural rearrangements sometimes include isochromosome 8q formation. Financial support for this project (to JAS) is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

3. B. Beheshti, J. Karaskova, B.G. Beatty, P.C. Park, J. Sweet, M. Beheshti, J. Trachtenberg, J.S. Rhim, B. Hukku, J.A. Squire, J.A. Macoska. Chromosomal Aberrations in Early Stage Prostate Cancer Patients and Virus-Immortalised Cell Lines Identified by Combined Comparative Genomic Hybridization, Spectral Karyotyping, and Allelotyping Techniques. January 30-Feb 1, 2000. Proceedings of the 8th International Workshop on Chromosomes in Solid Tumors, Tucson, AZ, USA; vol 8, p42.

   We have utilised comparative genomic hybridization (CGH) with confirmatory interphase nuclei fluorescence *in situ* hybridization (FISH) to examine fourteen early stage prostate cancer (CaP) patients for recurrent chromosomal copy number changes suggestive of tumor initiation and progression. In addition, combined conventional and spectral karyotyping (SKY) techniques and allelotype analysis were used to assess numerical and structural chromosomal alterations in two cell lines derived from normal human prostatic epithelium, and three

cell lines derived from primary human prostate tumor epithelium, immortalized with the E6 and E7 transforming genes of human papillomavirus (HPV) 16 or the Large T antigen gene of simian virus 40 (SV40). CGH and interphase FISH results suggested isochromosome 8q formation in the patient cohort. Interestingly, allelotyping identified loss of 8p sequences in two of the three primary prostate tumor-derived cell lines, and SKY analysis revealed that the loss of 8p sequences was directly due to isochromosome 8q formation and/or other structural alterations of chromosome 8. This provides evidence that 8p loss in primary human prostate tumors may, in some cases, result from complex structural rearrangements involving chromosome 8. Moreover, the data reported here provides direct evidence that such complex structural rearrangements sometimes include isochromosome 8q formation. Financial support for this project (to JAS) is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

4. J.A. Squire, B. Beheshti, L.R. Kapusta, L. Klotz, P.C. Park, Identification of early prognostic markers in CaP by subtractive hybridization and microarray analysis. January 30-Feb 1, 2000. Proceedings of the 8th International Workshop on Chromosomes in Solid Tumors, Tucson, AZ, USA; vol 8, p76.

   The difficulty in management of prostate cancer (CaP) stems largely from the paucity of information regarding the genetic events underlying prostate tumorigenesis. Currently 17-21% of low stage pT1/pT2 tumors progress to metastatic disease, while the remainder are indolent. A major challenge in CaP research therefore, is to identify prognostic markers that accurately predict outcome at the preinvasive phase. To this end, we applied suppression subtractive hybridization to compare the gene expression between two samples of prostate tumors (pT2; Gleason score 7) obtained from patients who present disease recurrence (n=3), and disease free survival (n=3) during five years of follow-up. Two normalized, reversely subtracted libraries were thus obtained and is presently undergoing characterization. Results to date include 13 individual, unique clones of which 5 have been identified as known genes, including a metalloproteinase, a DNA binding protein of unknown

function (chromosome 6), a DEAD box protein (17q23), a homeobox protein (15q14) and a BAC clone of chromosome 16 (16p11.2). Furthermore, the same cohort was screened using the Clontech Atlas Cancer array to examine the differences in expression levels of known genes. Increased gene expression associated with neovascularization, including VEGFR and VEGF was detected in this complimentary study. These results suggest that understanding the patterns of gene expression in early CaP lesions will be of prognostic value and will serve as the basis for the design of future CaP gene chips. PCP was supported by a grant from AUA/AFUD. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

5. P.C. Park, B. Beheshti, L.R. Kapusta, L. Klotz, J.A. Squire. Identification of early prognostic markers in CaP by subtractive hybridization and microarray analysis. Abstract accepted in the American Association for Cancer Research 91st Annual Meeting, San Francisco, CA, USA. April 2000.

   The difficulty in management of prostate cancer (CaP) stems largely from the paucity of information regarding the genetic events underlying prostate tumorigenesis. Currently 17-21% of low stage pT1/pT2 tumors progress to metastatic disease, while the remainder are indolent. A major challenge in CaP research therefore, is to identify prognostic markers that accurately predict outcome at the preinvasive phase. To this end, we applied suppression subtractive hybridization to compare the gene expression between two samples of prostate tumors (pT2; Gleason score 7) obtained from patients who present disease recurrence (n=3), and disease free survival (n=3) during five years of follow-up. Two normalized, reversely subtracted libraries were thus obtained and is presently undergoing characterization. Results to date include 13 individual, unique clones of which 5 have been identified as known genes, including a metalloproteinase, a DNA binding protein of unknown function (chromosome 6), a DEAD box protein (17q23), a homeobox protein (15q14) and a BAC clone of chromosome 16 (16p11.2). Furthermore, the same cohort was screened using the Clontech Atlas Cancer array to examine the differences in expression

levels of known genes. Increased gene expression associated with neovascularization, including VEGFR and VEGF was detected in this complimentary study. These results suggest that understanding the patterns of gene expression in early CaP lesions will be of prognostic value and will serve as the basis for the design of future CaP gene chips. PCP was supported by a grant from AUA/AFUD. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

6. B. Beheshti, B.G. Beatty, P.C. Park, J. Karaskova, J. Sweet, M.A.S. Jewett, M. Beheshti, and J.A. Squire. Chromosomal abnormalities associated with aggressive prostate cancer as identified by interphase FISH, comparative genomic hybridization and spectral karyotyping. Proceedings of the American Association for Cancer Research. March 1999. 40:235.

   To distinguish between the indolent and aggressive forms of prostate cancer (CaP), a better understanding of the chromosomal basis for prostate cell transformation and tumourigenesis is critical. We have applied comparative genomic hybridization (CGH) and spectral karyotyping (SKY) to 3 CaP cell lines representing advanced CaP, and 14 early stage pT1/pT2 CaP tumours in order to identify consistent chromosomal rearrangements and/or copy number changes that may be predictive of aggressive disease. SKY and CGH analyses demonstrated substantially more complex chromosomal rearrangements, and chromosomal gains and losses respectively in the hormone-independent lines DU145 and PC-3 (> 11 reciprocal or non-reciprocal translocations, at least 8 complex translocations involving multiple partner chromosomes, and several deletions) compared to either the hormone-dependent LNCaP cells or the fresh tumours. Breakpoints involving chromosomes 1, 4, 6, 10, and 15 were found in both LNCaP and either DU145 or PC-3 suggesting they may be early indicators of aggressive disease. In light of the high number of aberrations detected by SKY and CGH in advanced disease and the relatively normal CGH profiles obtained in the early stage fresh tumours, we suggest that clonal aneuploidy may be a feature of aggressive CaP. Preliminary data including identification of trisomy 8 in a subpopulation of CaP cells by interphase

FISH, implicate cellular heterogeneity and/or chromosomal instability as intrinsic features of early stage CaP tumours. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

7. B. Beheshti, B.G. Beatty, J. Bayani, J. Sweet, M.A.S. Jewett, P.C. Park, and J.A. Squire. Chromosomal abnormalities associated with aggressive prostate cancer as identified by interphase fluorescence *in situ* hybridization (FISH), comparative genomic hybridization (CGH) and spectral karyotyping (SKY). 54th Annual Canadian Urological Association Meeting. June 1999. Can. J Urol., 6(3):775.

   To distinguish between the indolent and aggressive forms of prostate cancer (CaP), a better understanding of the chromosomal basis for prostate cell transformation and tumourigenesis is critical. We have applied spectral karyotyping (SKY) and Giemsa-banding (G-SKY) to 3 CaP cell lines representing early and late stage CaP, and comparative genomic hybridization (CGH) to 14 early stage pT1/pT2 CaP tumours in order to identify consistent chromosomal rearrangements and/or copy number changes that may be predictive of aggressive disease. G-SKY analysis on the cell lines demonstrated substantially more complex chromosomal aberrations in the hormone-independent lines DU145 and PC-3 (> 11 reciprocal or non-reciprocal translocations, at least 8 complex translocations involving multiple partner chromosomes, and several deletions) compared to the hormone-dependent LNCaP cells. Consensus breakpoint regions involving the pericentric regions 1p10-p22, 4q10-q21, 6q10-q15, 10q10-q21, were found in all three cell lines suggesting they may be early indicators of aggressive disease. A comparison of DU145 and PC-3 aberrations by G-SKY identified ten common chromosomal breakpoints involving the centromeres 4, 5, 6, 8, 11, 12, 14, 15, and the consensus regions 3q13.1-q21 and 14q31-32. Despite these common aberrations detected by G-SKY in cell lines, the CGH profiles for the early stage fresh tumours were relatively normal. This suggests that clonal aneuploidy may be a feature of aggressive CaP. Preliminary data including identification of trisomy 8 in a subpopulation of CaP cells by interphase

fluorescence *in situ* hybridization (FISH), implicate cellular heterogeneity and/or chromosomal instability as intrinsic features of early stage CaP tumours. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

8. B. Beheshti, B.G. Beatty, J. Bayani, J. Sweet, M.A.S. Jewett, P.C. Park, and J.A. Squire. Chromosomal abnormalities associated with aggressive prostate cancer as identified by interphase FISH, comparative genomic hybridization and spectral karyotyping. (3rd prize poster; University of Toronto Laboratory Medicine and Pathobiology Student Research Day, February and May 1999).

   To distinguish between the indolent and aggressive forms of prostate cancer (CaP), a better understanding of the chromosomal basis for prostate cell transformation and tumourigenesis is critical. We have applied comparative genomic hybridization (CGH) and spectral karyotyping (SKY) to 3 CaP cell lines representing advanced CaP, and 14 early stage pT1/pT2 CaP tumours in order to identify consistent chromosomal rearrangements and/or copy number changes that may be predictive of aggressive disease. SKY and CGH analyses demonstrated substantially more complex chromosomal rearrangements, and chromosomal gains and losses respectively in the hormone-independent lines DU145 and PC-3 (> 11 reciprocal or non-reciprocal translocations, at least 8 complex translocations involving multiple partner chromosomes, and several deletions) compared to either the hormone-dependent LNCaP cells or the fresh tumours. Breakpoints involving chromosomes 1, 4, 6, 10, and 15 were found in both LNCaP and either DU145 or PC-3 suggesting they may be early indicators of aggressive disease. In light of the high number of aberrations detected by SKY and CGH in advanced disease and the relatively normal CGH profiles obtained in the early stage fresh tumours, we suggest that clonal aneuploidy may be a feature of aggressive CaP. Preliminary data including identification of trisomy 8 in a subpopulation of CaP cells by interphase FISH, implicate cellular heterogeneity and/or chromosomal instability as intrinsic features of early stage CaP tumours. Financial support for this

project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

9. B. Beheshti, B.G. Beatty, J. Bayani, J. Sweet, M.A.S. Jewett, P.C. Park, and J.A. Squire. Identification of common chromosomal rearrangements in pericentric regions of prostate cancer cell lines by sequential Giemsa-banding, spectral karyotyping, and interphase FISH. (2rd prize poster; Hospital for Sick Children Department of Paediatric Laboratory Medicine Research Day, June 1999).

To distinguish between the indolent and aggressive forms of prostate cancer (CaP), a better understanding of the chromosomal basis for prostate cell transformation and tumourigenesis is critical. We have applied comparative genomic hybridization (CGH) and spectral karyotyping (SKY) to 3 CaP cell lines representing advanced CaP, and 14 early stage pT1/pT2 CaP tumours in order to identify consistent chromosomal rearrangements and/or copy number changes that may be predictive of aggressive disease. SKY and CGH analyses demonstrated substantially more complex chromosomal rearrangements, and chromosomal gains and losses respectively in the hormone-independent lines DU145 and PC-3 (> 11 reciprocal or non-reciprocal translocations, at least 8 complex translocations involving multiple partner chromosomes, and several deletions) compared to either the hormone-dependent LNCaP cells or the fresh tumours. Breakpoints involving chromosomes 1, 4, 6, 10, and 15 were found in both LNCaP and either DU145 or PC-3 suggesting they may be early indicators of aggressive disease. In light of the high number of aberrations detected by SKY and CGH in advanced disease and the relatively normal CGH profiles obtained in the early stage fresh tumours, we suggest that clonal aneuploidy may be a feature of aggressive CaP. Preliminary data including identification of trisomy 8 in a subpopulation of CaP cells by interphase FISH, implicate cellular heterogeneity and/or chromosomal instability as intrinsic features of early stage CaP tumours. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

10. J. AL-Maghrabi, A. Toi, J. Sweet, S. Jothy, J. Trachtenberg, W. Chapman, M. Jewett, D. Benerjee, J.A. Squire. Role of numeric chromosomal changes detected by interphase FISH in high-grade prostrate intraepithelial neoplasia (HPIN) diagnosed in prostate needle core biopsies as a predictor of carcinoma. University of Toronto Laboratory Medicine and Pathobiology Student Research Day, March 2000.

HPIN is the most likely precursor of prostate cancer and its identification in biopsy specimens warrants further searching for invasive carcinoma; however not all cases of HPIN progress to cancer or associated with it. About half of HPIN showed carcinoma in the second subsequent follow-up biopsies. No available clinical or immunohistochemical or morphological criteria that can be predictive of this progression or association. Retrospective study on prostate biopsies diagnosed as HPIN with available follow-up subsequent biopsies. Two groups of patients identified. The first revealed carcinoma on the follow-up biopsies and the second revealed persistent HPIN and/or other benign lesions. Molecular cytogenetic analysis has been performed to determine if there is any difference in chromosomal instability between these two groups. Interphase FISH analysis was performed on biopsies from 28 patients (12 from the first group and 16 from the second). We have utilized formalin fixed paraffin-embedded tissue sections (5μ). Biotin labeled cosmid probe for chromosome 8 centromere and directly labeled VYSIS CEP probes for chromosome 4,7,10. Dual-probe hybridization has been performed. The number of signals has been counted in one hundred non-overlapped nuclei. The criteria for chromosomal gain and loss is >8% of cells with >2 signals and >50% of cells with <2 signals respectively. Thirty three percent of the first group displayed numerical chromosomal aberrations to a various degree. Only 12.5% of the patients from the second group had chromosomal anomalies. All chromosomal changes were detected in a form of gain and no chromosomal losses have been identified. Overall, the commonest anomaly was gain of chromosome 8, followed by chromosomes 7 and 10. No anomalies have been seen in the adjacent hyperplastic or normal prostate glandular epithelium. These results indicate that there is no single numeric chromosomal anomaly could be assigned

as a predictor of progression of HPIN to carcinoma. Although there is no statistically significant difference between the two samples ($P>0.05$) in the overall numeric chromosomal abnormalities for chromosomes 7, 8 and 10, however, our sample size is small and a larger sample size might be detective of a significant difference. Financial support for this project is funded by the U.S. Army Medical Research and Materiel Command (USAMRMC) Prostate Cancer Research Program (PCRP).

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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